The Journal of Laboratory and Clinical Medicine

VOL. 28 DECEMBER, 1942 No. 3

# CLINICAL AND EXPERIMENTAL

## EPINEPHRINE AND EPHEDRINE ANALOGUES AND THEIR CLINICAL ASSAY

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DISCOVERY of the chemical structure of epinephrine by Abel (1897) and its synthesis by Stolz (1904) and Dakin (1905) have been followed by the synthesis of many derivatives possessing pharmacologic actions resembling those of epinephrine.

The early synthesis of sympathomimetic amines by Barger and Dale, gave impetus to this study. They studied aliphatic amines with no attached benzene ring. Amines with less than four carbon atoms had little activity. Those with from four to six had some pharmacologic action. Addition of the benzene ring to a two-carbon chain, with two carbon atoms separating the ring from the nitrogen produced clear-cut sympathomimetic action (phenylethylamine, Table I). The introduction of hydroxyl groups at the para or meta positions within the ring resulted in more effective epinephrine-like action.

Epinephrine has hydroxyl groups in both meta and para positions. This arrangement in the ring constitutes the catechol nucleus. We may describe epinephrine as a compound formed by the union of the catechol nucleus with a two-carbon aliphatic amine in which hydroxyl is attached to the proximal carbon and a methyl radical to the nitrogen.

It was natural to assay those compounds which closely resembled epinephrine in terms of the latter as the standard. The most easily measured epinephrine effect is elevation of the blood pressure. In early pharmacologic studies, epinephrine derivatives were graded according to pressor activity and its duration, with pressor response to epinephrine designated as unity. Pressor action was found to be controlled in great measure by the hydroxyl groups in

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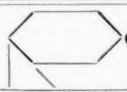
Received for publication, May 1, 1942.

the benzene ring. Tyramine differs from phenylethylamine only in the presence of a para hydroxyl group. The pressor activity of the former is double that of the latter. The addition of another hydroxyl group again doubles the ability of the compound to increase blood pressure (Table I, *4*). It will be noted in the last column of Table I that, as pressor activity increases, its duration becomes shortened.

TABLE I

| CHEMISTRY AND PHARMACOLOGY OF SOME ADRENALIN-LIKE COMPOUNDS |  | | | | | PRESSOR ACTIVITY (ADRENALIN-1) | PRESSOR DURATION (ADRENALIN-1) |
|---|---|---|---|---|---|---|---|
| 1. Phenylethylamine (PE) | H | H | H | H | H | 1/200-1/80 | 3-4 |
| 2. Tyramine | OH | H | H | H | H | 1/100-1/20 | 2 |
| 3. p-methoxy-PE | $OCH_3$ | H | H | H | H | 1/300 | 4 |
| 4. Dihydroxy-PE | OH | OH | H | H | H | 1/35 | 1 |
| 5. Epinine | OH | OH | H | H | $CH_3$ | 1/12 | 2 |
| 6. Arterenol | OH | OH | OH | H | H | 3/2 | 2 |
| 7. Epinephrine | OH | OH | OH | H | $CH_3$ | 1 | 1 |
| 8. Neosynephrin | H | OH | OH | H | $CH_3$ | | |
| 9. Paredrine | OH | H | H | $CH_3$ | H | | |
| 10. Benzedrine (beta-phenylisopropylamine) | H | H | H | $CH_3$ | H | 1/300-1/100 | 5-10 |
| 11. Propadrine | H | H | OH | $CH_3$ | H | 1/300-1/60 | 7 |
| 12. Ephedrine | H | H | OH | $CH_3$ | $CH_3$ | 1/300-1/100 | 7 |
| 13. Corbasil | OH | OH | OH | $CH_3$ | H | 1/4 | 2 |
| | Para | Meta | Beta | Alpha | | | |

The pharmacologic activity of epinephrine (at least as regards its pressor effect) appears dependent upon the catechol nucleus. The nucleus apparently accounts also for the short pressor duration since it is rapidly oxidized. Efforts have been made to counteract this rapid oxidation. Methylation of the hydroxyl radical increases stability but causes loss in pressor effectiveness (Table I, *3*).

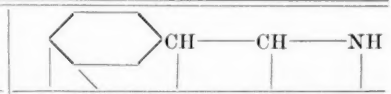
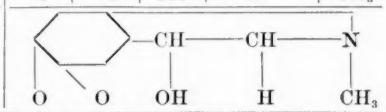
For better orientation we may review the prevailing hypotheses concerning the synthesis and destruction of epinephrine. There are gaps in the experimental evidence. The hypotheses cannot be considered proved, but for simplicity we shall present them didactically, because the theories are those most generally favored by chemists and pharmacologists and because they present a sequential picture, facilitating clinical discussion. Until there are better hypotheses, progress must be based on those now in favor.

Phenylalanine seems to be the precursor of epinephrine. It is an amino acid indispensable for growth. The possible succession of chemical changes brought about by enzymes is illustrated in Table II. The introduction of one hydroxyl in the benzene ring transforms phenylalanine into tyrosine, another amino acid. A second hydroxyl introduction produces dihydroxyphenylalanine. Next, the beta carbon is oxidized, after which the amino group is methylated. Decarboxylation of the alpha carbon produces epinephrine.

Destruction of epinephrine is an oxidative process, which can be brought about in two ways. In one, oxygen replaces the hydrogen of the hydroxyls in the catechol nucleus, producing an unstable quinone of epinephrine. Further internal oxidation reduction and molecular rearrangement result in an unstable indole derivative called adrenochrome, in which the nitrogen is attached to the benzene ring. Adrenochrome is inactive and appears responsible for the red brown discoloration of oxidized epinephrine.

This auto-oxidative process probably occurs in vitro. While it may occur in vivo, there appears to be a second process, incited by an enzyme, amine oxidase, which attacks many amines, converting them into aldehydes and ammonia (or in the case of epinephrine, into aldehydes and methylamine). Amine oxidase effect is comparable to that of cholinesterase against acetylcholine. Probably epinephrine is not destroyed too rapidly in the tissues because of the protective action of amino acids, which serve as hydrogen donors for epinephrine. Glutathione and ascorbic acid also serve as reducing substances. Both are abundant in the adrenal glands and probably participate in stabilization of epinephrine in the gland and in the blood leaving it.

TABLE II

| SYNTHESIS AND OXIDATION OF EPINEPHRINE | |  | | | | |
|---|---|---|---|---|---|---|
| | | | | CH—CH—NH | | |
| Synthesis | 1. Phenylalanine | H | H | H | COOH | H |
| | 2. Tyrosine | OH | H | H | COOH | H |
| | 3. Dihydroxphenylalanine | OH | OH | H | COOH | H |
| | 4. Oxidation of beta carbon | OH | OH | OH | COOH | H |
| | 5. Methylation of amino group | OH | OH | OH | COOH | $CH_3$ |
| | 6. Decarboxylation | OH | OH | OH | H | $CH_3$ |
| Oxidation | 7. Quinone of epinephrine | O | O | OH | H | $CH_3$ |
| | 8. Adrenochrome |  | | | | |
| | | | | CH—CH—N | | |
| | | O | O | OH | H | $CH_3$ |

Briefly summarizing, the pressor activity of epinephrine and probably its other sympathomimetic effects are due primarily to the presence of hydroxyl groups in the benzene ring. This catechol nucleus also accounts for the evanescence of the response, epinephrine being rapidly oxidized by amine oxidase. The effectiveness of related synthetic compounds is also due to hydroxyl groups in the benzene ring.

Impetus was given the study of other synthetic compounds of potential clinical value by the identification of ephedrine (Table I). Ephedrine differs from epinephrine in the absence of hydroxyl groups in the benzene ring and the presence of a third carbon atom in the aliphatic chain in the form of a methyl group. Ephedrine effect is similar to that of epinephrine, but it does differ from it. Without the oxidizable catechol nucleus, ephedrine is not as easily broken down and is, therefore, effective when taken by mouth. For the same reason, its pressor activity is less, but the duration of pressor action is lengthened. Other physiologic effects, such as relief of bronchospasm, are similarly less pronounced.

Today there are over 200 compounds related to epinephrine and ephedrine which might have some sympathomimetic action. Of those related to ephedrine we shall use as illustrations only the four indicated in Table I. These illustrate the outstanding differences between the epinephrine group and the ephedrine group, summarized as follows:

In the absence of the catechol nucleus, the compounds are not destroyed as quickly.

The methyl group in the alpha carbon position prolongs pressor action but diminishes its intensity.

This methyl group may be responsible for the central nervous stimulation.

The addition of a phenolic hydroxyl group reduces the central stimulation. Paredrine has an arrangement in the benzene ring similar to that of tyramine, while its side chain is similar to benzedrine. It does not stimulate the nervous system. Corbasil is nonstimulating.

Ephedrine and related compounds, unlike epinephrine, produce tachyphylaxis. Successive doses given at short intervals exert progressively diminishing effect.

Gaddum's theory of the action of ephedrine, propadrine, benzedrine, and related compounds is an attractive hypothesis. Amine oxidase destroys epinephrine by oxidation. Ephedrine, like epinephrine, has an attraction toward amine oxidase. Indeed, its attraction is greater. Ephedrine combines with amine oxidase, thereby protecting the epinephrine normally present in the body. Consequently, epinephrine appears to become more active. Ephedrine potentiates epinephrine by blocking amine oxidase. Ephedrine being slowly oxidized, persists in combination with amine oxidase.

This would explain why small doses of ephedrine appear to increase the activity of epinephrine. Without having made quantitative studies, we have recognized the apparent increased effectiveness of an ephedrine-epinephrine combination as compared with straight epinephrine in clinical work and have used such a combination routinely for the past ten years. Our preparation consists of equal parts of 1:1,000 epinephrine solution and 3 per cent aqueous ephedrine. The dose of the combination has been the same as the dose of 1:1,000 epinephrine alone, usually 0.3 c.c. of the mixture.

Large doses of ephedrine sometimes appear to do the reverse, blocking epinephrine effect. The explanatory theory is that ephedrine, like epinephrine, may combine with the chemicals of the sympathetic myoneural junction. Ephedrine exerts no sympathomimetic action itself, and in this chemical combination it blocks epinephrine, obstructing the activity of the latter. With our small clinical doses this has not been a problem.

A suggested explanation of the central stimulation of benzedrine (Mann and Quastel, 1940) is based on the theory that these compounds combine with amine oxidase, blocking its activity. As stated above, the oxidation of certain amines releases aldehydes. Aldehydes exert an inhibiting effect on cerebral respiration (oxidation). Benzedrine binds amine oxidase so that it cannot oxidize the amines. Toxic aldehydes are not liberated. As a consequence cerebral respiration is less impeded.

## CLINICAL APPLICATION

From time to time new synthetics of this series are proposed for clinical use. The first question usually is, "Is it as good as epinephrine or ephedrine?" The tendency is to weigh the effectiveness of any other compound in terms of these two. Propadrine, benzedrine, and neosynephrin are compared with ephedrine. Proprietary nebulizers with solutions having special trade names such as Vaponefrin or Neosol are presented, sometimes with the innuendo that the solution is superior to epinephrine. On questioning, one is usually informed

that the material is alpha methylamino-beta-hydroxyl dihydroxybenzene. Study of Table I indicates that this is epinephrine. For general sympathomimetic effect, nothing has as yet been found superior to epinephrine.

We do not imply that no such compound may eventually be found. Gunn has formulated four criteria for the ideal sympathomimetic: (1) it should be effective when given orally; (2) its action should be more prolonged than that of epinephrine; (3) it should be more certain than ephedrine; (4) there should be no central stimulation. The nearest approach in our past experience has been the mixture of epinephrine and ephedrine, which, however, still requires hypodermic administration.

Though the ideal sympathomimetic is not yet at hand, it may be that one compound is superior for one type of effect, while others are better for other actions. This is true with propadrine, benzedrine, neosynephrin, and paredrine. The question should not be "Are they as good as epinephrine or ephedrine," but rather, "Do they excel these in one or another activity, and do they have any superiority in lessened undesirable side effects?"

Epinephrine, for example, is effective in shrinking turgescent nasal mucosa. In fact, it is too effective, producing so much vasoconstriction with tissue anoxia that secondary vasodilatation may ensue, with consequent increased edema. One to 3 per cent ephedrine produces local decongestion, usually adequate, and not enough to cause anoxia. Neosynephrin appears to have special value here in that it is effective in much lower concentration (0.25 per cent). Although benzedrine produces undesirable central stimulation, its base is volatile. This gives it definite value as an inhalant in nasal allergy.

On the other hand, neosynephrin exerts little action when taken internally, except in large doses. It is about as effective as ephedrine in relaxing bronchospasm, but for adequate dosage it must be given hypodermically and is therefore inferior to ephedrine, although it has a slight superiority in that it exerts less nervous stimulation. Although benzedrine dilates the bronchi, this effect is not marked, and there is little change in respiratory rate, minute volume, or vital capacity. There is no unanimity of opinion concerning its action on gastrointestinal musculature.

We see therefore that neosynephrin and benzedrine base are superior compounds for topical application but possess no outstanding superiority with regard to other effects in the allergic diseases. The value of benzedrine in narcolepsy might be mentioned parenthetically, although this is not in the category of allergic diseases.

Paredrine exerts little action on the bronchi or gastrointestinal tract. It has pronounced pressor action and is not stimulating to the nervous system. Its outstanding effectiveness is as a mydriatic.

Propadrine exerts less central stimulation than ephedrine or benzedrine, has about the same pressor activity as ephedrine, and is about as effective when applied to the nasal mucosa. It may be given by mouth in the same dose or double the dose of ephedrine. Propadrine therefore finds use with patients who find ephedrine too stimulating.

Many other compounds have been studied, including kephrine, synephrin, arterenol, paredrinol, sympatol and epinine. Since none have superior effectiveness in relieving local or general allergic reactions, we shall not discuss them.

The list of compounds which might be superior in one or another respect is not complete, and the desirability of continuing the search is obvious, even though one must realize that a majority of the related compounds may be less effective than those now available.

## PLAN OF INVESTIGATION

On the assumption that certain compounds may show superiority in relieving one or another but not necessarily all allergic responses, clinical investigation should include assay of sympathomimetic and allied responses in all tissues that lend themselves to objective investigation. Our preliminary program was outlined as follows:

*Increased Capillary Permeability.* A compound which will diminish capillary hyperpermeability, basically characteristic of the allergic response, should be of value, provided no undesired side effects accompany this action. Objective study might include the action of drugs upon experimentally induced histamine and allergen wheals. Epinephrine or ephedrine, or both, should be used as standards for comparison.

Localized subcutaneous edema of the angioneurotic type might be studied in its response to sympathomimetic drugs by determination of tissue tension in the edematous area (Vaughan and Pipes, 1940).

Both methods were employed, but it was found that tissue tension changes in wheals were not always sufficiently pronounced for reliable evaluation, and that the problem of producing angioneurotic edema at will in predisposed persons could not be easily solved. Tissue tension studies were therefore discontinued.

The action of compounds on whealing was studied in both normal and allergic persons.

*Bronchial Obstruction.* The obstructive phenomenon in bronchial asthma is probably due to local submucosal edema as well as bronchospasm. In the present study it is unimportant which is the dominant factor, since the desired therapeutic result, bronchial dilatation, would hold in either case.

Alexander and Kountz and others have shown that epinephrine and ephedrine increase vital capacity in asthmatic and in normal persons. Starr, also Richards, Barach and Cromwell suggest that maximum ventilation in liters per minute may be a better index of improved pulmonary function than vital capacity.

At the beginning, vital capacity studies were made. Later these were supplemented with ventilation capacity determinations.

*Local Vasomotor Response.* Here again it is unimportant whether the reaction in the nose is due to increased capillary permeability or vascular dilatation, or both. The therapeutic criterion is increased ventilation through the nose.

We have found the most satisfactory measure of nasal mucosal shrinkage in the Glatzel-Mulinos nasal mirror. This gives roughly quantitative results. Our adaptation of this method has been described elsewhere (Vaughan and Derbes, 1941).

*Gastrointestinal Hypertonicity.* There is no generally accepted objective method for clinically determining the response of smooth muscle in the intes-

tines. The methods used have yielded conflicting results (e.g., in the case of benzedrine). It seemed fruitless to repeat these procedures. Subjective tests were made with some persons subject to repeated gastrointestinal allergic reactions, manifested especially by distention. These patients tried different preparations repeatedly, not knowing which were being given, and expressed their conclusions as to which were more effective. We found propadrine and ephedrine most effective. Propadrine was preferred since it produced less jitters.

*Toxic Allergic Fatigue.* In our experience a phase of pronounced fatigue is an occasional accompaniment of food allergy, especially if the allergenic food is not rapidly removed by vomiting or diarrhea. This fatigue may appear within from thirty minutes to several hours, and usually lasts for twenty-four hours or longer. Certain persons appear to be especially susceptible to this response.

Here, again, any evaluation must be subjective. Two persons who were subject to allergic fatigue and were well acquainted with the manner of their response to allergenic foods evaluated different compounds in terms of comparable relief from 5 mg. of benzedrine. Benzedrine was most effective and had least unpleasant side effect in 5 mg. dosage. Propadrine hydrochloride, 25 mg., and neosynephrin hydrochloride, 10 mg. capsules, were somewhat less effective but had a definite benzedrine-like stimulating action and no unpleasant side effects. Beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride,* 50 mg. capsules, was slightly lower in stimulating action than propadrine. Ephedrine caused little stimulation and much jitters.

It is regrettable that no adequate objective measures of therapeutic benzedrine effect are available. This drug in usual dosage produces no consistent or diagnostic change in metabolic rate, blood sugar level, or deep reflexes, or in the formed elements of the blood. Evaluation must be made in terms of elevation of mood, euphoria, lessened fatigue, enhanced motor and speech activity, feeling of increased efficiency and increased ability to concentrate, irritability and sleeplessness.

*Undesired Effects.* Notations were made of undesired responses, when present, such as palpitation, tremor, "jitteriness," blood pressure elevations, tachycardia, urinary retention, etc.

## ORIENTATION STUDIES

A preliminary survey was made to determine whether the contemplated methods would be sufficiently sensitive. This evaluation included study of the blood pressure, pulse rate, and vital capacity and notation of the presence or absence of palpitation or jitters. Increase in the number of leucocytes and erythrocytes per cubic millimeter of blood was also determined (Table III).

We started with methyl racephedrine and beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride† (Table IV). These were compared with epinephrine and ephedrine.

*This will hereafter be simplified to read, "methoxyphenyl-propyl*di*methylamine." The italicized -di- serves to differentiate this compound from a similar mono- compound which will be mentioned subsequently in this report.

†The analogues studied were prepared according to the methods described by Woodruff, Lambooy, and Burt.

Three tenths cubic centimeter of 1:1,000 epinephrine subcutaneously produced an average increase in blood pressure of 24 mm., an increase in pulse rate of 11, and an average increase in vital capacity of 272 c.c. This was usually accompanied by palpitation and jitters.

Ephedrine, 25 mg. subcutaneously, caused an average rise of 12 mm. blood pressure, an increase in pulse rate of 5, and an increased vital capacity of 167 c.c. This also usually caused jitters.

TABLE III

| RESPONSE TO SUBCUTANEOUS EPINEPHRINE AND ANALOGUES | BLOOD PRESSURE INCREASE | PULSE RATE INCREASE | LEUCOCYTE INCREASE | ERYTHROCYTE INCREASE | AVERAGE INCREASE IN VITAL CAPACITY | PALPITATION AND JITTERS |
|---|---|---|---|---|---|---|
| Epinephrine 1:1,000 0.3 c.c. | 24 | 11 | 2,600 | 150 M | 272 | Yes |
| Ephedrine 25 mg. | 12 | 5 | 700 | 300 M | 167 | Yes |
| Methyl racephedrine 25 mg. | 0 | 0 | None | 200 M | 74 | No |
| Beta-o-methoxyphenyl-n-propyldimethyl-amine hydrochloride 25 mg. | 0 | 0 | None | 0 | 74 | No |

Methyl racephedrine and methoxyphenyl-propyl*di*methylamine caused no increase in blood pressure or pulse rate and no jitters. They did produce an increased vital capacity, less, however, than that produced by ephedrine (Table III). The second preparation caused a slight decrease in blood pressure.

These studies were made without knowledge of the previous laboratory results with animals. They were then compared. The correspondence between laboratory and clinical results appeared to be satisfactory. The pressor activity of ephedrine had been found to be from 1/100th to 1/250th that of epinephrine, while methyl racephedrine was 1/4,000th and methoxyphenyl-propyl*di*methylamine had been slightly depressor in animals. The correlation in bronchial relaxation was not as good. We found an average increase in vital capacity after methyl racephedrine of only 74 c.c.; and after methoxyphenyl-propyl*di*methylamine, 74 c.c. Isolated lung tests had indicated that the two new compounds, especially the latter, might be superior to ephedrine in relaxing bronchoconstriction. We had given them in the same dosage as ephedrine (25 mg. subcutaneously).

The preliminary studies indicate that in identical dosage neither of the substitutes is as effective as ephedrine in increasing vital capacity. Further, none of the three is as effective as epinephrine. However, two possibilities were obvious. First, the new drugs might be as effective as ephedrine, or more so, if given in larger doses. Toxicity studies indicate that much larger dosage may be safely employed (Table IV). If in larger quantities they do not produce jitters as does ephedrine, they might have value in therapy. The second possibility was based on the absence of pressor response and tachycardia. Some effective drug may be found which in adequate dosage causes no increase in blood pressure.

Five subjects were used in each vital capacity series, four normals and one asthmatic. Ephedrine produced increased vital capacity in all subjects.

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Epinephrine failed to produce increase in one subject, and the two ephedrine analogues failed in two and three subjects, respectively.

The one asthmatic in the series had an increase of 400 c.c. after epinephrine, 70 c.c. after ephedrine, and none after methyl racephedrine or methoxyphenyl-propyl*di*methylamine.

The second phase of the orientation study involves disappearance of histamine wheals after medication. Wheals were produced by the injection of 0.03 c.c. of 1:1,000 histamine hydrochloride. They were fully evolved after five minutes. We first determined the time required for involution of this wheal. Thereafter, the drugs were injected subcutaneously five minutes after the intracutaneous injection of histamine.

No more rapid involution was caused by 47.1 mg. of racephedrine than in the controls, while 94.2 mg. of racephedrine caused as rapid involution as after 0.3 c.c. of 1:1,000 epinephrine. Subjective symptoms were as pronounced after 94 mg. of racephedrine as after epinephrine. Palpitation and jitters persisted for four hours. At the end of four hours another histamine wheal was produced. This wheal was smaller than the original, indicating an inhibiting effect from 94.2 mg. of racephedrine at the end of four hours.

We next compared the involution after 50 mg. of ephedrine with that after 46.4 mg. of methyl racephedrine and 45.8 mg. of methoxyphenyl-propyl-*di*methylamine. Methyl racephedrine was less effective than ephedrine. The second product, in slightly lower milligram dosage, was comparable to ephedrine.

This observation, if substantiated by a larger series, might suggest a specific function of methoxyphenyl-propyl*di*methylamine in the control of allergic diseases associated with whealing or increased capillary permeability, especially since in comparable dosage (46 mg. vs. 50 mg.) less side effect (palpitation and jitters) was noted than after ephedrine.

The final phase of the orientation study consisted in therapeutic trial, giving the two preparations in capsule form to persons with active allergic symptoms.

Fifty-two patients with various allergic symptoms were given methyl racephedrine, 51 patients received methoxyphenyl-propyl*di*methylamine (each in 25 mg. dosage by mouth). Of these, 34 were given both. Not all reported results. Thirty-two reported on the former, 35 on the latter, and 22 on both.

Of the 32 receiving methyl racephedrine, 19 reported subjective improvement and 2 described unpleasant symptoms. Of the 35 receiving methoxyphenyl-propyl*di*methylamine, 25 were helped and 2 reported unpleasant symptoms.

Eleven reported equal relief from both drugs, seven had no relief from either, three concluded that methyl racephedrine was more effective, five that methoxyphenyl-propyl*di*methylamine was more effective, and 14 concluded that the drugs were equally beneficial (Table V). The drugs were equally efficacious in asthma and rhinitis. Methoxyphenyl-propyl*di*methylamine was possibly more effective in urticaria.

These patient experiences appeared to confirm the objective studies in indicating that the drugs had some effectiveness in relieving bronchospasm and that the second was more effective in the relief of whealing.

TABLE IV

| CHEMISTRY OF COMPOUNDS STUDIED |  | | | | | | | ACUTE TOXICITIES (MG. PER KG.) | | | CHRONIC TOXICITIES (MG. PER KG.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | INTRAVENOUS IN RATS | INTRAVENOUS IN RABBITS | ORAL IN RATS | ORAL IN RATS | | ORAL IN RABBITS | |
| | | | | | | | | | | | DOSE | MORTALITY | DOSE | MORTALITY |
| Ephedrine | H | H | OH | $CH_3$ | $CH_3$ | H | | 110 | 70 | 400 | 250 | 50% | 100 | 0 |
| | | | | | | | | | | | 100 | 0 | | |
| Methyl racephedrine | H | H | OH | $CH_3$ | $CH_3$ | $CH_3$ | | 90 | | | 250 | 0 | 100 | 0 |
| Beta-o-methoxyphenyl-n-propyldimethylamine hydrochloride | H | $OCH_3$ | $CH_3$ | H | $CH_3$ | $CH_3$ | | 40 | | | 250 | 0 | 100 | 0 |
| | | | | | | | | | | 1,100 | | | | |
| | | | | | | | | | | 500 | | | | |

At the termination of these orientation studies, we believed that we had evolved a reliable technique for clinical pharmacologic study with which to study these and other ephedrine-like drugs in varying dosage using larger series of subjects.

TABLE V

RESULTS OF CLINICAL TRIAL

| DRUGS* (ORALLY) | CASES HELPED | CASES NOT HELPED | SUBJECTIVE SYMPTOMS | CASES HELPED BY BOTH DRUGS | CASES HELPED BY NEITHER DRUG | NO. 1 BETTER THAN NO. 2 | NO. 1 AND NO. 2 EQUALLY GOOD | NO. 2 BETTER THAN NO. 1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 19 | 13 | 12 | 11 | 7 | 3 | 14 | 5 |
| 2 | 25 | 10 | 12 | | | | | |

RESULTS BY SYMPTOMS

| SYMPTOM AND NUMBER OF CASES | DRUG* (ORALLY) | CASES | CASES HELPED | CASES NOT HELPED | SUBJECTIVE SYMPTOMS | CASES GIVEN BOTH DRUGS | CASES HELPED BY BOTH | CASES HELPED BY NEITHER | NO. 1 BETTER THAN NO. 2 | NO. 1 AND NO. 2 EQUALLY GOOD | NO. 2 BETTER THAN NO. 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Asthma | 1 | 20 | 12 | 8 | 2 | 12 | 5 | 4 | 2 | 8 | 2 |
| 26 | 2 | 18 | 12 | 6 | 1 | | | | | | |
| Rhinitis | 1 | 7 | 5 | 2 | 0 | 6 | 4 | 1 | 1 | 3 | 2 |
| 13 | 2 | 12 | 10 | 2 | 0 | | | | | | |
| Urticaria | 1 | 7 | 4 | 3 | 0 | 5 | 3 | 1 | 0 | 3 | 2 |
| 7 | 2 | 5 | 4 | 1 | 0 | | | | | | |
| Angioneurotic | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| edema 1 | 2 | 1 | 0 | 1 | 1 | | | | | | |
| Migraine | 1 | 0 | | | | 0 | | | | | |
| 1 | 2 | 1 | 1 | 0 | 0 | | | | | | |

*Drug 1 = Methyl racephedrine hydrochloride.
Drug 2 = Beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride.

ASSAY OF EPINEPHRINE AND EPHEDRINE ANALOGUES

The program of clinical assay as we have developed it includes determination of the pulse rate, blood pressure, and vital capacity before and after hypodermic administration of the test drug, and the effect of the injected material on the evolution or involution of histamine wheals produced either by iontophoresis or intracutaneous injections. In this series we did not study the local effect on the nasal mucosa.

In some of the series vital capacity determinations were supplemented by determinations of the coefficient of ventilation, as described by Brice. The coefficient of ventilation represents the ratio of oxygen consumed per minute to the oxygen respired for a minute. Theoretically, this test has an advantage over the vital capacity test, since, in the former, the patient rests passively, while in the latter active cooperation is required. Determination of the coefficient of ventilation is as follows:

The patient reports in at the same time each day, but not necessarily in the postabsorptive state. After a twenty-minute rest in bed a tracing is made

similar to that for the basal metabolic rate determination. We used the Jones metabolism apparatus. The usual line touching the points of expiration is drawn, as well as a line touching the points of inspiration. The distance between the lines measures the mean respiratory amplitude (MRA) or tidal ventilation. The time required to consume one liter of oxygen is determined, and from this the cubic centimeters of oxygen consumed per minute ($O_2C$) is calculated. The average respiratory rate (ARR) is obtained by counting the total respirations and dividing this total by the time required for the test. The coefficient of ventilation is then calculated from the formula:

$$CV = \frac{O_2C \times 100}{ARR \times MRA}$$

After completion of the tracing and recording of pulse rate and blood pressure, the patient stands next to the bed and blows into a spirometer for measurement of the vital capacity, after which he again lies down. These determinations are repeated after twenty minutes, after which the drug to be tested is injected subcutaneously. The four determinations, pulse rate, blood pressure, coefficient of ventilation, and vital capacity, are then repeated at twenty-minute intervals for an additional two hours and twenty minutes. The total time required is three hours. To avoid the possibility of cumulative effect, only one drug is tested each day. Each subject was tested with each of the drugs under study.

The response to 0.4 c.c. of 1:1,000 epinephrine as a standard for comparison was studied in nine cases. In seven, determinations were made of response to 50 mg. of ephedrine, 25 mg. of ephedrine, 50 mg. of methyl racephedrine hydrochloride, 50 mg. of beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride, and 50 mg. of beta-o-methoxyphenyl-n-propyl-methylamine hydrochloride.* The time required was not inconsiderable, amounting to 44 seances of three hours each. The results are summarized in Table VI B, which is derived as follows:

The actual determinations are averaged, as illustrated for the case of epinephrine in Table VI A. The first vertical column on Table VI B is derived from Table VI A by subtracting the higher of the two control figures from the highest of the several average figures recorded after the injection of epinephrine. Figures for the other drugs were derived from tables similar to Table VI A.

Epinephrine increased the vital capacity an average of 460 c.c. as compared with 120 c.c. for ephedrine, and 20, 70, and 30 c.c. each for the analogues tested. The coefficient of ventilation was increased most after epinephrine, while the analogues compared favorably with ephedrine in this respect.

As a check on the reliability of these objective methods of study of respiratory efficiency, clinical trial was made by dispensing 25 mg. capsules of the analogues to 70 patients with asthma, 35 with rhinitis, and 19 with urticaria for comparison with each other and with 25 mg. dosages of ephedrine. In no case was the effectiveness superior to that of ephedrine, and in the majority all three analogues were less effective. In comparing the three among themselves some patients believed that one was a little better, while other patients

*See footnote,* page 261.

selected another. While there was no uniformity beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride was selected a little more often as better than the other two in both asthma and urticaria. Furthermore, it depressed experimental histamine whealing definitely better than the other two, and indeed, slightly more effectively than did ephedrine in the same dosage.

It is our impression that the increase in vital capacity after these several drugs more nearly paralleled their actual effectiveness in relieving asthma than did the change in the coefficient of ventilation. This is fortunate to the extent that vital capacity determination is much less time-consuming than ventilation coefficient determination.

TABLE VI A

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF 0.4 C.C. OF EPINEPHRINE ON THE PULSE RATE, BLOOD PRESSURE, VITAL CAPACITY, AND COEFFICIENT OF VENTILATION (AVERAGE OF 9 CASES)

| | CONTROL | | MINUTES AFTER INJECTION OF EPINEPHRINE | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 20 | 40 | 60 | 80 | 100 | 120 | 140 |
| Pulse rate | 81.3 | 81.3 | 81.4 | 81.9 | 81.1 | 79.4 | 78.9 | 78.0 | 80.0 |
| Blood pressure | 118.8 | 120.1 | 128.0 | 125.8 | 125.8 | 122.0 | 117.6 | 119.9 | 120.9 |
| Vital capacity | 2.49 | 2.60 | 2.99 | 3.06 | 3.0 | 3.01 | 2.74 | 2.63 | 2.66 |
| Coefficient of ventilation | 2.42 | 2.58 | 3.46 | 3.39 | 3.43 | 3.96 | 3.98 | 4.20 | 4.48 |

TABLE VI B

AVERAGE INCREASE IN PULSE RATE, BLOOD PRESSURE (MM. HG), VITAL CAPACITY (LITERS) AND COEFFICIENT OF VENTILATION AFTER SUBCUTANEOUS TREATMENT WITH TEST DRUGS

| | EPINEPHRINE 1:1,000 0.4 C.C. | EPHEDRINE 50 MG. | EPHEDRINE 25 MG. | METHYL RACEPHEDRINE 50 MG. | BETA-O-METHOXYPHENYL-N-PROPYL-*di*-METHYLAMINE 50 MG. | BETA-O-METHOXYPHENYL-N-PROPYLMETHYLAMINE 50 MG. |
|---|---|---|---|---|---|---|
| Pulse rate | 0.6 | 3 | 1.1 | 0.1 | 0.3 | 1.2 |
| Blood pressure | 7.9 | 10.5 | 3.2 | 11.3 | 5.6 | 11.9 |
| Vital capacity | 0.46 | 0.12 | 0 | 0.02 | 0.07 | 0.03 |
| Coefficient of ventilation | 1.90 | 0.41 | 0.87 | 0.92 | 0.76 | 0.69 |

As a final check we ran another series of vital capacity determinations on an additional six cases, comparing the response to ephedrine, methyl racephedrine, and beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride, given subcutaneously. Beta-o-methoxyphenyl-n-propylmethylamine hydrochloride was not used in this series, since it had been found clinically to be the least effective of the three. The average increase after ephedrine was 190 c.c.; after methyl racephedrine, 120 c.c.; and after beta-o-methoxyphenyl-n-propyl*di*methylamine hydrochloride it was 250 c.c. This shows the same superiority of the last over methyl racephedrine. When this last series is compared with the results in Table VI B there is a consistency in the findings, indicating that vital capacity determination is an adequate measure of the effectiveness of these drugs in asthma.

Beta-o-methoxyphenyl-n-propyl-*di*methylamine hydrochloride was no more effective clinically than ephedrine, and yet in the last series it was more effective in increasing the vital capacity to the extent of 60 c.c. We conclude that a difference of at least 60 c.c., probably better 75 or 100 c.c. must be present to be significant.

## CONCLUSIONS

A program has been devised for the clinical assay of ephedrine analogues. This consists in serial determinations (before and after hypodermic administration of the drug) of pulse rate, blood pressure, and vital capacity; determination of the drug's effect on the evolution and involution of histamine wheals; and study of the local reaction in one side of the nose compared with the other side as a control, with the use of the Glatzel-Mulinos Mirror, as modified by Vaughan and Derbes.

Both epinephrine and ephedrine should be used as comparison controls. Superiority over ephedrine should require an average increase of vital capacity of at least 75 c.c. better than ephedrine.

Results of such objective study should be checked by clinical trial.

Failure of an ephedrine or epinephrine analogue to benefit one allergic symptom or reaction does not necessarily mean that it will be ineffective in all such reactions. Clinical assay should, therefore, evaluate the several types of response.

The drugs studied were provided through the courtesy of The Upjohn Research Laboratories where preliminary pharmacologic laboratory investigations had been made.

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# THE EFFECT OF TESTOSTERONE PROPIONATE IN THE TREATMENT OF ARTERIOSCLEROSIS OBLITERANS*

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THE effects of testosterone on the vascularity of the skin[1] and on muscle tissue metabolism[2, 3] have suggested its use in the treatment of patients with arteriosclerosis obliterans. This is a report of a preliminary study made with the intention of evaluating results in a small series of cases to determine the advisability of further work along these lines.

We studied 23 patients; eight of these were treated with biweekly intramuscular injections of 25 mg. of testosterone propionate; fifteen patients were control cases, receiving biweekly intramuscular injections of 3 c.c. of normal saline. All patients were ambulatory and had had a complete general and peripheral vascular study. The peripheral vascular status was determined on the basis of that study.[4] The patients had received no previous treatment for peripheral vascular disease. All were instructed as to foot hygiene and care, diet, and general measures, as outlined in our clinic routine. All patients were re-examined and studied at approximately three- to six-month intervals, and their peripheral vascular status was determined according to the following criteria:

A. Vascular anatomic status: This was determined by the evaluation of the amplitude of vessel pulsation determined by palpation, oscillometry, temperature changes, rubor on dependency and pallor on elevation, and roentgenograms for arterial calcification. This factor was graded from four plus (severe) to zero (no involvement).

B. Tissue anatomic status: This was determined by the degree of involvement of the superficial and deep tissues, and graded four plus when gangrene was present, three plus when ulceration was present, two plus when infection without gangrene was present, one plus when skin atrophy muscle atrophy, nail changes, or epidermatophytosis were present, and zero when no apparent involvement was present.

C. Rest pain: This refers to pain, other than that of claudication, and includes neuritic pain, paresthesias, coldness, night cramps. This was graded four plus for severe pain, to zero for no pain.

D. Claudication: This was measured by the patient's statement regarding the number of blocks he could walk before pain in calves supervened. He was graded four plus if he could walk only one-half block; three plus if he could

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Received for publication, June 26, 1942.

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walk one-half to two blocks; two plus, if two to four blocks; one plus, if more than four blocks, but still limited; zero, if no claudication was present.

E. Vascular reserve or capacity for vasodilatation: This was studied by means of the thermal vasodilatation test.[5] Where this test did not produce complete vasodilatation, nerve block was performed for corroboration.[6] Vascular reserve was graded four plus for full dilatation; i.e., a rise in skin surface temperature of the big toe to 30.5° C.; three plus, a 75 per cent rise from the initial temperature to 30.5° C.; two plus, a 50 per cent rise; one plus, a 25 per cent rise; zero, no rise.

F. Functional classification: This represents a summation of the disease factors. Class I, patients with organic vascular disease without symptoms; Class IIa, patients with organic vascular disease with minimal symptoms; Class IIb, patients with organic vascular disease with moderately severe symptoms; Class III, patients with organic vascular disease bedridden because of gangrene, infection, or intractable pain; Class IV, patients with symptoms, but with no demonstrable organic vascular disease.

We are reporting the results of our findings at the end of a three- to seven-month period, an eight- to fourteen-month period, and a fourteen- to eighteen-month period of treatment.

## RESULTS

After treatment for three to seven months, four patients treated with testosterone propionate showed no improvement in vascular status; two of these patients were improved in tissue anatomic status, rest pain was lessened in two cases, claudication was improved in two cases, vascular reserve was improved in two cases, and functional status was improved in two cases.

Of the seven control cases observed for this period, four cases were improved in vascular anatomic status; two cases were improved in tissue anatomic status, rest pain; and claudication time were not improved in any case; three cases were improved in vascular reserve; two cases were improved in functional status.

It will be noted that for this period of study results were equivocal.

After eight to fourteen months of treatment with testosterone propionate, none of the six cases studied showed improvement in vascular anatomic status, two were improved in tissue anatomic status, rest pain was unrelieved in any case, four were improved in claudication time, three were improved in vascular reserve, and four were improved in functional status.

Of six control cases, two improved in vascular anatomic status, none improved in tissue anatomic status, none was relieved of rest pain, two were improved in claudication time, three were improved in vascular reserve, and two were improved in functional status.

It will be noted that, for this period, no encouraging results were obtained.

After fourteen to eighteen months of treatment with testosterone propionate, one of the two cases studied was improved in vascular anatomic status, one improved in tissue anatomic status, one in claudication time; rest pain was not present, both improved in vascular reserve, and one improved in functional status.

Of the six control cases, two improved in vascular anatomic status, two improved in tissue anatomic status, none were relieved of rest pain, three were improved in claudication time, in vascular reserve, and in functional status.

It will be noted that, for this period of treatment, results were not encouraging.

SUMMARY

Twenty-three patients suffering from obliterative vascular disease of the lower extremities were studied to note the effect of testosterone propionate on the signs and symptoms of their disease. Eight cases received biweekly intramuscular injections of 25 mg. of testosterone propionate; fifteen patients were observed as controls. The entire study was carried on over a period of eighteen months.

No significant effect was noted in the treated cases with respect to vascular anatomic status, tissue anatomic status, vascular reserve, claudication, rest pain, or functional status, as compared with the control cases.

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# A STUDY OF NORMAL CARDIAC RESPONSE TO WATER BELOW BODY TEMPERATURE WITH SPECIAL REFERENCE TO A SUBMERSION SYNDROME*

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IN MANY institutions the importance of being able to swim as a means of self-preservation is felt so keenly that everyone is required to acquire an elementary mastery of it. Wherever large groups are required to go into a swimming pool, there are always some who experience reactions so disturbing that they never have a feeling of well-being when submerged† in water. In fact, in some cases, their reactions to submersion are so pronounced that it may appear hazardous to compel them to undertake to learn to swim.

These special cases always place an instructor in a quandary as to what to do. It is always a question whether the person who appears distressed when

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Received for publication, June 29, 1942.

†Submersion as used here does not necessarily mean that a person is completely under water. In all cases the head is out of the water.

submerged in water merely is afraid, or whether he actually has physiologic reactions which might be hazardous were he left unsupervised while attempting to swim.

Each year there are numerous drownings attributed to heart failure, cramps, and exhaustion. Horton and Gabrielson[1] investigated the actual causes and concluded that in all probability many drownings were due, in reality, to a syndrome which they called "hypersensitiveness to cold." As a result of their investigations, they propose two tests which seem adequate for detecting persons who are hypersensitive to cold. By making use of such tests it is proposed that persons who are hypersensitive to cold may be appraised of the fact and can thus avoid unpleasant experiences due to submersion in cold water.

The tests proposed by Horton and Gabrielson are (1) the ice-cube test, and (2) the cardiovascular test. The ice-cube test is the simpler of the two but is less reliable because the systemic reactions are limited. It is done as follows: a cube of ice is held in contact with the forearm of a person in the sitting position for three minutes. If he is hypersensitive to cold, a wheal slightly raised and surrounded by a red flare will appear within two to five minutes after the removal of the ice cube.

The cardiovascular test is more complicated, but recommended, because it gives better systemic reactions. The test consists of establishing resting heart rates and blood pressures in the supine position. Then the hand and forearm of the subject are immersed in water at a temperature of 8° to 10° C. for from five to ten minutes. At the end of five minutes of immersion blood pressure and pulse rate are recorded. Then the subject's hand is removed from the bath, and blood pressure and pulse rate are taken every two minutes for the next fifteen to twenty minutes. At the same time observations are made for swelling, hives, color changes, and subjective sensations.

In hypersensitive persons exposure of the hand and forearm to a cold bath causes a definite decrease both in systolic and diastolic blood pressures, accompanied by an increase in pulse rate. Other symptoms of hypersensitiveness are swelling of the submerged hand and a definite mark of demarcation at the water line.

The reactions of hypersensitive people to cold are said to be due to a release of a chemical substance, histamine, from the tissue cells of the body in sufficient concentration to produce histamine reactions. In fact, the reactions of the hypersensitive person can readily be duplicated by the injection of histamine.

A number of studies have been reported on the effects of submersion in water on the physiologic reactions both of diving and land animals.[2] The results of these investigations parallel, to a considerable extent, those obtained in the case of man. When diving animals go from land into the water, they suffer a marked drop in heart rate, provided they are accustomed to the surrounding environment. However, when land animals are forcibly submerged, they do not show a similar reaction. The diving animals are observed to relax in the water, while the land animals fail to do so. Due to the unnatural environment even animals show physiologic, as well as psychologic, behavior which is dangerous to their existence in water.

The reactions of some animals, as well as some human beings, to even partial submersion in water raise the question: Should people be forced promiscuously into swimming pools, even though it is granted that swimming is a fine sport, a healthy exercise, and a useful thing to be able to do?

Researches dealing with water submersion show conclusively that man, when even partially submerged, experiences alterations in physiologic responses, especially those involving the cardiovascular system.

Wells[3] studied the effect of submersion in water of varying temperatures on heart rate. His technique was to place the subject in a supine position in a specially arranged tub in which water temperature could be controlled. He began with a temperature of 65° F. and gradually raised it to 110° F. The heart rate was counted at every 5 degree change in temperature. The results of this study are shown in Table I.

TABLE I

EFFECT OF SUBMERSION IN WATER OF VARYING TEMPERATURES ON THE HEART RATE OF 46 NORMAL MALE SUBJECTS

| WATER TEMPERATURE DEGREES F. | HEART RATE PER MINUTE |
|---|---|
| 65 | 72 |
| 70 | 61 |
| 75 | 68 |
| 80 | 66 |
| 85 | 65 |
| 90 | 64 |
| 95 | 64 |
| 100 | 70 |
| 105 | 87 |
| 110 | 120 |

The data in Table I show that there is a decrease in heart rate due to submersion until the water temperature approximates the body temperature. From this point on, there is an increase in heart rate as long as there is an increase in the water temperature. Since the temperature of swimming pools is maintained somewhere between 65° and 85° F., it is reasonable to expect that persons who respond normally will show a decrease in heart rate when submerged in a swimming pool.

The results of an experiment by Tuttle and Corleaux[4] are further evidence that the normal reaction to submersion in water is a significant decrease in heart rate. They compared the heart rates of 100 men sitting and standing in their normal environment with their pulse rates sitting and standing when submerged in water. The results of this experiment are shown in Table II. The figures are group means. The rates are for one minute.

TABLE II

MEAN PULSE RATES OF A GROUP OF 100 MEN BEFORE AND DURING SUBMERSION IN WATER OF SWIMMING POOL TEMPERATURE

| | MEAN NORMAL PULSE RATE | MEAN PULSE RATE IN POOL | MEAN CHANGE IN PULSE RATE |
|---|---|---|---|
| Sitting | 87 | 76 | -11 |
| Standing | 98 | 84 | -14 |

The data in Table II are further evidence that the normal reaction to submersion in water of swimming pool temperature is a decrease in pulse rate.

In seeking to establish the response of normal persons to submersion in water, it has been our experience that there has always been a small percentage of cases who failed to conform to the reactions of the group as a whole. It is the purpose of this investigation to study the reactions of this group who appear to react abnormally to submersion.

In order to have available nonconformants for further study a group of persons, unskilled in swimming, were examined in the class situation. Sixty-eight college women registered in beginning swimming classes at the University of Iowa served as subjects. Their ages ranged from 17 to 25 years.

Early in the instructional period each girl was tested as to the response of her heart to submersion. First, a resting pulse rate in the standing position was recorded. Following this, she climbed into the swimming pool and waded into water of shoulder depth. After three minutes her resting pulse rate was recorded again. The count was continued at two-minute intervals as long as there was a change in pulse rate, or until a constant rate was established.

Of the group of 68 women, 7 failed to show a decrease in heart rate while submerged in water and 61 responded normally. A summary of the data collected from the normal group is shown in Table III. The figures are group means per minute.

TABLE III

CARDIAC RESPONSE OF 61 NORMAL SUBJECTS TO SUBMERSION IN WATER

| MEAN RESTING RATE STANDING (PER MINUTE) | MEAN RESTING RATE STANDING—SUBMERGED (PER MINUTE) | DIFFERENCE |
|---|---|---|
| 90 | 77 | -13 |

The data in Table III show that, on the average, there was a decrease of 13 beats per minute, which is practically the same as that reported for men. The mean decrease amounts to 14 per cent for women as compared to 12 per cent for men.

Table IV shows the relationship between resting pulse rate and the decrease caused by submersion.

TABLE IV

RELATION OF RESTING PULSE RATE TO THE DECREASE IN RATE CAUSED BY SUBMERSION IN WATER

| RESTING PULSE RATE (PER MINUTE) | DROP DUE TO SUBMERSION |
|---|---|
| 70-79 | 5 |
| 80-89 | 11 |
| 90-99 | 14 |
| 100-109 | 16 |

The data show that the extent of the decrease in pulse rate due to submersion in water varies directly with the resting pulse rate.

The group of 7 girls who failed to respond normally to submersion in water were studied further. There were two possible explanations for the abnormal response of this group: (1) there was lack of psychologic adjustment; that is, they were afraid. In the case of adjustment to submersion in water, fright alone is sufficient to account for a failure to adjust normally; that is, experience a fall in heart rate. (2) There was lack of physiologic adjustment.

Ordinarily, one can expect that repetition of exposure to a strange environment will condition a person so that he will exhibit normal behavior. On the other hand, it is reasonable to suppose that if abnormal response is due to the failure of some physiologic mechanism to adjust itself, repetition will fail to cause an appreciable alteration in the response.

The submersion experiment was repeated on the 7 subjects who failed to experience a decrease in heart rate during submersion. In the interim between the first test and the retest, each member of this group had participated in six or more class hours of swimming instruction, during which they were submerged in water. One would expect this amount of repetition to condition a person to the new environment. The results of the retest are shown in Table V.

TABLE V

RESULTS OF A RETEST OF SUBJECTS WHO FAILED TO SHOW NORMAL REACTIONS TO SUBMERSION IN WATER

| SUBJECT NO. | | RESTING PULSE RATE (PER MINUTE) | PULSE RATE WHILE SUBMERGED (PER MINUTE) | DIFFERENCE |
|---|---|---|---|---|
| 18 | First test | 82 | 80 | - 2 |
| | Retest | 86 | 74 | -12 |
| 32 | First test | 92 | 90 | - 2 |
| | Retest | 96 | 90 | - 6 |
| 53 | First test | 78 | 78 | 0 |
| | Retest | 80 | 76 | - 6 |
| 23 | First test | 68 | 82 | +14 |
| | Retest | 72 | 76 | + 4 |
| 28 | First test | 80 | 80 | 0 |
| | Retest | 72 | 72 | 0 |
| 43 | First test | 86 | 90 | + 4 |
| | Retest | 82 | 92 | +10 |
| 56 | First test | 86 | 88 | + 2 |
| | Retest | 88 | 88 | 0 |

The data in Table V show that 3 of the 7 subjects originally classed as abnormal became conditioned to submersion. Subject 18 behaved as a psychologic case, and after sufficient exposure to a water environment responded normally. Subjects 32 and 53 became conditioned to the water to such an extent that there was a significant fall in pulse rate due to submersion.

However, 4 of the 7 cases (23, 28, 43, and 56, Table V) failed completely to become conditioned. They never experienced a decrease in heart rate due to submersion, and it was suspected that they were failing to make normal physiologic adjustments to submersion.

Although the water in the swimming pool was at a temperature between 75° and 85° F. and could not be considered cold, it was decided to subject the nonconditioned group to a test for hypersensitiveness to cold. The cardiovascular function test was given to each, and all of them were hypersensitive to cold according to the criteria suggested by Horton and Gabrielson.

Although it would seem that this group of persons had been exposed to submersion a sufficient number of times so that they should be psychologically conditioned to it, it was decided to gather additional data relative to this point.

For this purpose, each of the subjects (23, 28, 43, and 56) was given a bathtub test. The procedure was to place each person in a bathtub in a sitting position and determine the resting heart rate. Then she sat at the side of the tub while it was filled with water of the temperature of that of the swimming pool. She returned to the tub and after three minutes of submersion the resting pulse rate was taken again. Under these conditions the resting pulse rate was established as previously described while the subject was submerged. The data obtained are shown in Table VI.

TABLE VI

EFFECT ON HEART RATE OF SUBJECTS BEING SUBMERGED IN WATER IN A BATHTUB

| SUBJECT NO. | RESTING PULSE RATE (PER MINUTE) | PULSE RATE SUBMERGED (PER MINUTE) | DIFFERENCE |
|---|---|---|---|
| 23 | 66 | 68 | +2 |
| 28 | 68 | 70 | +2 |
| 43 | 66 | 66 | 0 |
| 56 | 64 | 64 | 0 |

The data in Table VI show results similar to those obtained in the previous experiments. The data show that, although these subjects did not experience as great an increase in heart rate when submerged in the tub as in the pool, they failed to show any tendency toward a decrease. This lack of physiologic adjustment on the part of some persons may account for their being uncomfortable in the swimming pool, and also the difficulty experienced by them in learning to swim.

## CONCLUSIONS

On the basis of data collected from 68 college women, the following conclusions are drawn concerning the effects of submersion in water:

1. Submersion in water of swimming pool temperature causes a drop in the heart rate of normally adjusted persons.

2. The amount of the decrease in heart rate due to submersion varies directly with the resting heart rate.

3. Failure to experience a decrease in heart rate when submerged in water below body temperature is due either to a lack of emotional adjustment (fear) or to a failure to compensate physiologically.

4. Where emotional factors are controlled, failure to experience a significant drop in pulse rate during submersion in water below body temperature indicates sensitivity to the water.

5. It is suggested that the conditions causing a failure to make normal adjustments to submersion in water be called the "submersion syndrome."

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# ETIOLOGY AND SERUM TREATMENT OF PERSISTENT EPIDEMIC AND POSTOPERATIVE HICCUP*

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PREVIOUS reports have been made[6, 7, 9, 11] on the consistent isolation from atria of infection of patients suffering from epidemic hiccup, of alpha or green-producing streptococci with which spasms of the diaphragm and other muscles (myoclonic encephalitis) have been produced experimentally in animals. Paroxysms of spasms of the diaphragm, sometimes associated with audible hiccup, were reproduced consistently by direct injection of suspensions of nasopharyngeal swabbings and filtrates of these and with pure cultures of the living streptococcus freshly isolated in dextrose-brain broth, as well as with the corresponding washed, heat and formalin-killed organisms and with filtrates of active cultures.[8] Characteristic symptoms developed in from twenty-four to seventy-two hours after inoculation of relatively small numbers of the living streptococci, in from four to twelve hours after inoculation of large numbers of the killed organisms, and in two to five hours after inoculation of Berkefeld N filtrates. The symptoms after inoculation of the living streptococci lasted the longest and often progressed to death in forty-eight to ninety-six hours; those that occurred after inoculation of the dead streptococci lasted usually from six to twenty-four hours, and those following inoculation of filtrates lasted two to six hours and then disappeared.

Cultural characteristics, virulence, cataphoretic velocity, and antigenicity of the streptococci resembled those of streptococci isolated in studies of epidemic encephalitis. Vaccines prepared from the spasm-producing streptococci protected rabbits against living cultures, the dead organisms, and active filtrates.[8] The streptococci were agglutinated specifically by the encephalitis antistreptococcic serum. This antiserum was found to neutralize specifically the "toxin" or poison produced by the streptococcus of persistent hiccup and to have striking curative effects, under controlled conditions, in animals in which spasms of the diaphragm were produced experimentally.[8] Moreover, the encephalitis antistreptococcic serum appeared curative in the treatment of patients suffering from epidemic and postoperative hiccup.[5, 9]

The occurrence of encephalitis and hiccup simultaneously or in sequence and the experimental production of lesions in animals, corresponding to those responsible for characteristic symptoms in patients, with the respective streptococci freshly isolated from atria of infection of patients having diverse manifestations during a changing epidemic from hiccup to neuromyeloencephalitis,[10] indicate that the inciting agent of these diseases is similar.[1-3, 10] Moreover, the

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Received for publication, June 29, 1942.

changes in localizing power of the streptococci, as isolated from persons, and in this study also from milk and air, on artificial cultivation and on repeated passage through animals indicate that the changes in type of disease in this and other outbreaks studied were due to acquired specificities of streptococci normally present in atria of infection of well persons and in nature generally in nonepidemic times.

The study of the problem of epidemiologic characteristics, causation, and serum treatment of persistent epidemic hiccup has been extended to that of persistent postoperative hiccup. In this new series of cases, as in those previously reported, the cases studied culturally, and those in which serum was used in treatment, occurred chiefly among patients of the Mayo Clinic. The historical aspects of this strange disease, its classification, its reproduction in animals, and its results of serum treatment in a previous series of cases have been set forth by C. W. Mayo. I am indebted to members of the staff and to physicians in widely separated regions of the United States for their cooperation in permitting me to study cases and for reports of results from the use of the serum in cases of intractable hiccup.

## METHODS

A diagnostic cutaneous test[4] was made by injecting intradermally on the forearm of the patient 0.03 c.c. of a 10 per cent solution of the euglobulin fraction of antiserum prepared with the streptococci isolated in studies of encephalitis and, as controls, the euglobulin of other antisera. The size of the maximal flare of erythema, which occurred in from five to ten minutes, was outlined with pen and ink and from this the area of erythema in square centimeters was determined.

Precipitation tests[4] were made with the whole encephalitis and other antistreptococcic sera and cleared extracts of nasopharyngeal swabbings and the serum of patients, and agglutination tests[4] were made with the respective antisera and convalescent sera and the streptococci as isolated and after passage through animals. Dilutions of 1:10, 1:50, 1:250, and 1:1,250 of the respective antistreptococcic sera, and dilutions of 1:20, 1:40, 1:80, and 1:160 of convalescent and control human sera were used in agglutination tests. Mixtures of respective diluted sera and suspensions of streptococci that had been grown in dextrose-brain broth and preserved in dense suspension in glycerin (2 parts) and 25 per cent solution of sodium chloride (1 part) were incubated at 50° C. for eighteen to twenty-four hours when readings were made, instead of incubating at 37° C. for one and a half hours and then placing in the refrigerator overnight.

Material for cultures and inoculation of animals was obtained chiefly from swabbings of nasopharynges, from pus expressed from tonsils or aspirated from pyorrhea pockets, from urine, from pus expressed from prostates, from samples of stool, and in postoperative cases, in addition, from localized infections, if present, such as appendiceal abscess and infected gall bladder. Cultures were made on blood agar plates and in dextrose-brain broth.

Routinely two rabbits, in special instances as many as ten rabbits per case, and in some instances guinea pigs, were inoculated intracerebrally with 0.1 and

0.2 c.c., respectively, of the suspension of material directly from the nasopharynx, tonsils, and so forth, suspended in 2 c.c. of gelatin (0.2 per cent) Locke's solution, or with these amounts of pure cultures of the streptococcus in dextrose-brain broth, diluted in dextrose-brain broth 1:200 or 1:10,000 immediately before injection. In special instances, much higher dilutions (as high as 1:100,000,000) of cultures were given intracerebrally, and undiluted cultures were given intravenously and intratibially to rabbits. Monkeys and dogs were inoculated intracerebrally with from 1 to 5 c.c. of cultures diluted 1:200 to 1:10,000. Five-tenths cubic centimeter to 1 c.c. of suspensions of the heat-killed streptococci, ten times the density of broth cultures, or of filtrates of cultures, was inoculated intracerebrally into rabbits.

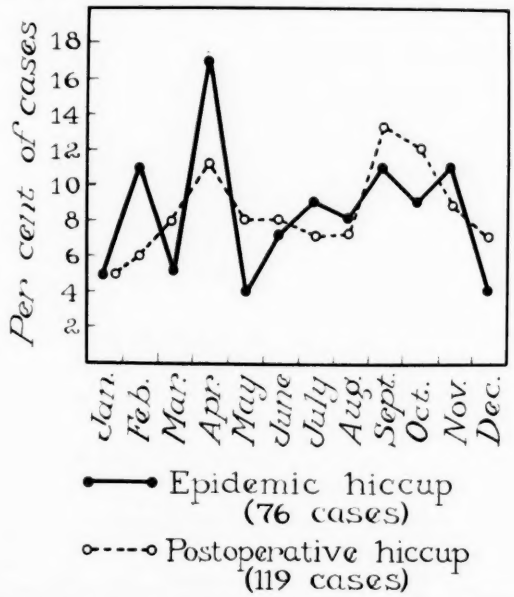


Fig. 1.—Percentage incidence by months of cases of epidemic and postoperative hiccup, Mayo Clinic, 1927 to 1941.

Pure cultures of the streptococcus were obtained from mixtures by making subcultures in rapid succession in previously warmed dextrose-brain broth, and from the end point of growth in serial dilution cultures[12] alternately in dextrose-brain broth and soft dextrose-brain agar (0.2 per cent dextrose and 0.2 per cent agar) and not by plating on blood agar. One or a few additional platings on blood agar usually destroyed the property on which elective localization depends, whereas this property was maintained at the end point of growth of streptococci in serial dilution cultures and after as many as 75 rapidly repeated subcultures in dextrose-brain broth.

Animals were observed two or more times daily after inoculation and their symptoms were recorded. Necropsies were performed as soon as possible after death from the inocula or from ether. Cultures from pipettings of the brain, spinal cord, cerebrospinal fluid, and blood were made routinely in dextrose-brain broth and on blood agar plates. In special instances cultures were made from liver, spleen, kidney, adrenals, muscles, and nerves. Tissues were put aside in 10 per cent solution of formalin for microscopic study.

Cultures were made from the air of rooms occupied by persons having persistent hiccup by exposing open tubes of dextrose-brain broth or by bubbling the air through dextrose-brain broth, chick-mash medium, and dextrose broth for twenty-four to forty-eight hours. For precipitation tests sterile distilled water was likewise exposed in shallow layers in Petri dishes or air was bubbled through water contained in tall columns in test tubes.

### SEASONAL INCIDENCE OF EPIDEMIC AND POSTOPERATIVE HICCUP

The occurrence of cases of persistent hiccup in unusual numbers at certain seasons was noted throughout the years of this study (1927 to 1941). There were great variations in severity and duration of the disease and accompanying gastric distress and other symptoms. The great majority of cases studied occurred in men from 17 to 82 years of age. In most instances attacks followed mild, and less often severe, respiratory infections. The seasonal and simultaneous occurrence of epidemic and postoperative hiccup in relation to relatively mild outbreaks of respiratory infections in spring and autumn is well illustrated in Fig. 1.

TABLE I

ERYTHEMATOUS REACTIONS OF PERSONS HAVING PERSISTENT HICCUP TO INTRADERMAL INJECTION OF THE EUGLOBULIN FRACTION OF ENCEPHALITIS AND OTHER ANTISTREPTOCOCCIC SERA

| DISEASE GROUPS | CASES | ERYTHEMATOUS REACTIONS TO: EUGLOBULIN FROM STREPTOCOCCIC ANTISERA | | | | | | NORMAL HORSE SERUM |
|---|---|---|---|---|---|---|---|---|
| | | ENCEPHALITIS | POLIOMYELITIS | EPILEPSY | ARTHRITIS | INFLUENZA | ULCERATIVE COLITIS | |
| Epidemic and postoperative hiccup | 20 | 32* / **6:69** | 8 / 4:25 | 10 / 5:50 | 18 / 3:22 | 10 / 5:40 | 12 / 2:50 | 18 / 1:0 |
| Epidemic encephalitis | 32 | 66 / **7:68** | 20 / 5:55 | 8 / 6:63 | 25 / 5:36 | 19 / 5:53 | | 30 / 1:0 |
| Acute influenza | 32 | 25 / 1:20 | 7 / 1:0 | | 15 / 1:26 | 32 / **12:91** | 10 / 1:50 | 8 / 0.5:0 |
| Normal controls | 84 | 91 / 1:13 | 93 / 1:8 | | 81 / 2:13 | 77 / 1:17 | 135 / 1:7 | 84 / 1:0 |

*The figures above the line in each instance indicate the number of tests; the two sets of figures below the line indicate, respectively, the average reaction in square centimeters and the percentage of reactions measuring 5 sq. cm. or more. Fractions are given to the nearest whole number.

### RESULTS OF CUTANEOUS AND PRECIPITATION TESTS

The results from intracutaneous inoculation of persons having persistent epidemic or postoperative hiccup with solutions of the water insoluble or euglobulin fraction of the serum of horses immunized with streptococci isolated in studies of encephalitis, poliomyelitis, epilepsy, arthritis, influenza, and ulcerative colitis are summarized in Table I. The reactions indicate a close relation between the infecting agents of epidemic and postoperative hiccup and epidemic encephalitis. Both the average reaction and the percentage of reactions 5 sq. cm. or more in size in these two groups of cases were greatest to the encephalitis euglobulin, second greatest to the epilepsy euglobulin, third greatest to the influenza euglobulin, and least to the poliomyelitis and arthritis euglobulins. The average reaction and percentage of reactions of 5 sq. cm. or more in the group of cases of influenza were extremely high to the influenza

euglobulin and low to the other euglobulins. The average reaction and incidence of reactions of 5 sq. cm. or more to the different euglobulins were consistently low in the group of normal controls.

The results of precipitation reactions between the encephalitis and other antistreptococcic sera and cleared extracts of nasopharyngeal swabbings and sera of persons having epidemic or postoperative hiccup are summarized in Table II. In accord with epidemiologic and clinical observations, the incidence of positive reactions with sera and extracts of nasopharyngeal swabbings of patients having persistent hiccup was strikingly high with the encephalitis antiserum and moderately so with influenza and epilepsy antistreptococcic sera. Likewise, in the groups of cases of encephalitis and influenza, the reactions were high with the respective homologous antisera and consistently low in the control group of well persons.

TABLE II

PRECIPITATION REACTION BETWEEN ENCEPHALITIS AND OTHER ANTISTREPTOCOCCIC SERA AND CLEARED EXTRACTS OF NASOPHARYNGEAL SWABBINGS AND SERA OF PATIENTS

| ANTIGENS | DISEASE GROUPS | CASES | EXTRACTS OR SERA | PERCENTAGE INCIDENCE OF PRECIPITATION* BY STREPTOCOCCIC ANTISERA FOR | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | ENCEPHALITIS | POLIOMYELITIS | ARTHRITIS | INFLUENZA | ULCERATIVE COLITIS | EPILEPSY |
| Extracts of nasopharyngeal swabbings | Epidemic and postoperative hiccup | 22 | 26 | **61** | 23 | 35 | **65** | 54 | **73** |
| | Epidemic encephalitis | 114 | 114 | **78** | 21 | 5 | 0 | 0 | |
| | Acute influenza† | 70 | 70 | 16 | 14 | 34 | **69** | 20 | 34 |
| | Well persons remote from epidemics | 60 | 68 | 4 | 0 | 3 | 4 | 6 | 0 |
| Sera of patients | Epidemic and postoperative hiccup | 24 | 30 | **37** | 0 | 0 | **27** | 10 | **33** |
| | Epidemic encephalitis | 67 | 67 | **73** | 25 | 0 | 0 | 0 | |
| | Acute influenza | 22 | 21 | 0 | 0 | 8 | 22 | | |
| | Well persons remote from epidemics | 108 | 108 | 4 | 2 | 6 | 5 | 2 | 0 |

*No precipitation occurred with normal horse serum.

†The term "influenza" in this study is used to designate the more severe type of seasonal epidemic respiratory infections.

## AGGLUTINATION OF THE STREPTOCOCCUS

The results of agglutination tests with streptococci isolated from atria of infection in cases of epidemic and postoperative hiccup, encephalitis and influenza, and from well persons remote from epidemics are summarized in Table III. A high incidence of specific agglutination occurred with the encephalitis antistreptococcic serum and streptococci isolated from atria of infection of persons having persistent hiccup, and interestingly, the incidence was relatively high with antisera prepared with the closely related streptococci isolated in studies of influenza and the spasm-producing streptococci isolated in studies of epilepsy. Streptococci isolated in studies of encephalitis and influenza were specifically agglutinated by the respective homologous antisera, whereas control strains from well persons were not.

TABLE III

AGGLUTINATION BY ENCEPHALITIS ANTISTREPTOCOCCIC SERUM OF STREPTOCOCCI ISOLATED DURING STUDIES OF EPIDEMIC AND POSTOPERATIVE HICCUP

| SOURCE OF STREPTOCOCCI | CASES OR STRAINS | CULTURES | PERCENTAGE INCIDENCE OF SPECIFIC AGGLUTINATION* BY STREPTOCOCCIC ANTISERA FOR | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ENCEPHALITIS | POLIOMYELITIS | ARTHRITIS | INFLUENZA | ULCERATIVE COLITIS | EPILEPSY |
| Epidemic and postoperative hiccup | 40 | 49 | **45** | 0 | 0 | **16** | 0 | **39** |
| Epidemic encephalitis | 96 | 144 | **75** | 9 | 10 | 0 | 0 | 3 |
| Epidemic influenza | 84 | 84 | 0 | 5 | 5 | **57** | | 24 |
| Well persons remote from epidemics | 49 | 49 | 8 | 4 | 22 | 14 | 0 | 16 |

*No agglutination with normal horse serum occurred with any of the suspensions and in some agglutination was nonspecific.

As has been shown, encephalitis streptococcic antigen was demonstrated in the serum by precipitation tests (Table II) and in the skin by intradermal injection of antibody (Table I) during the acute stage of epidemic and postoperative persistent hiccup. As recovery occurred, promptly following therapeutic injection of the encephalitis antistreptococcic serum, and more slowly without serum treatment, the reactivity of serum and skin (antigen) disappeared as specific antibodies became demonstrable for strains of streptococci obtained in studies of hiccup and encephalitis. The results of agglutination tests with convalescent serum are well illustrated by the following observations.

Each of nine sera obtained from persons convalescent from hiccup agglutinated to a greater degree and in higher dilution seven of nine strains of streptococci isolated in studies of epidemic and postoperative hiccup than did nine control sera obtained from patients having arthritis, fibrositis, or influenza. The nine convalescent sera did not agglutinate composite suspensions of streptococci isolated in studies of arthritis, epilepsy, and influenza, respectively. In sharp contrast, the sera from persons having arthritis, epilepsy, or influenza agglutinated specifically the composite suspensions of streptococci isolated, respectively, in studies of these diseases.

## ANIMAL EXPERIMENTS

The results obtained on intracerebral inoculation of the living streptococcus, the heat-killed streptococcus, and filtrates of cultures of streptococci in relation to persistent hiccup are summarized in Table IV. It will be noted that the incidence of hyperpnea (49 to 100 per cent) and of spasms of the diaphragm (62 to 88 per cent) and other muscles (65 to 90 per cent) was extremely high, and the incidence of flaccid paralysis (0 to 13 per cent) was very low after inoculation of the living streptococci, corresponding dead streptococci, and filtrates of cultures obtained in relation to hiccup. In contrast, the incidence of spasms of the diaphragm (2 per cent) and other muscles (19 per cent) after inoculation of streptococci from well persons and from indoor air of rooms of persons having poliomyelitis (0 and 57 per cent, respectively) was low. The incidence of flaccid paralysis was extremely low (6 per cent) after inoculation of the streptococci from the group of well persons, and very high (81 per cent) after inoculation of streptococci from indoor air in relation to

TABLE IV

RESULTS IN RABBITS AFTER INTRACEREBRAL INOCULATION OF THE LIVING STREPTOCOCCUS, FILTRATES OF CULTURES, AND THE HEAT-KILLED STREPTOCOCCUS, ISOLATED IN STUDIES OF EPIDEMIC AND POSTOPERATIVE HICCUP

| SOURCE OF STREPTOCOCCI AND FILTRATES | | STRAINS | ANIMALS INJECTED | SYMPTOMS (PER CENT) | | | | ANIMALS THAT DIED | | | | | CULTURES YIELDING STREPTOCOCCI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | SPASMS | | | | | PER CENT WITH LESIONS OF: | | | BRAIN | | BLOOD | |
| | | | | HYPERPNEA | DIAPHRAGM | OTHER MUSCLES | FLACCID PARALYSIS | NUMBER | PER CENT | DIAPHRAGM | LUNG | STOMACH | CULTURES | PER CENT | CULTURES | PER CENT |
| Living streptococci from: Nasopharynx | Epidemic hiccup | 40 | 128 | 63 | 62 | 65 | 3 | 74 | 58 | 24 | 42 | 39 | 91 | 86 | 81 | 2 |
| | Postoperative hiccup | 35 | 89 | 49 | 71 | 81 | 0 | 47 | 53 | 15 | 62 | 34 | 63 | 73 | 39 | 5 |
| | Influenza during outbreak of hiccup | 18 | 21 | 90 | 62 | 67 | 5 | 19 | 90 | 32 | 68 | 21 | 12 | 100 | 12 | 67 |
| | Well persons remote from epidemic hiccup (controls) | 44 | 64 | 25 | 2 | 19 | 6 | 27 | 42 | 0 | 48 | 15 | 40 | 53 | 22 | 9 |
| | Indoor air, cases of epidemic hiccup | 5 | 15 | 80 | 73 | 87 | 0 | 13 | 87 | 0 | 62 | 31 | 12 | 75 | 12 | 0 |
| | Indoor air, cases of epidemic poliomyelitis | 10 | 21 | 29 | 0 | 57 | 81 | 18 | 86 | 0 | 67 | 0 | 15 | 73 | 13 | 0 |
| | Pasteurized and raw milk supplies, cases of persistent hiccup | 10 | 30 | 50 | 80 | 90 | 13 | 10 | 33 | 40 | 60 | 30 | 8 | 63 | 8 | 0 |
| Nasopharynx, postoperative hiccup | Filtrates of nasopharyngeal washings | 9 | 16 | 88 | 81 | 81 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Filtrates of dextrose-brain broth cultures | 7 | 10 | 90 | 70 | 70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Suspensions of heat-killed streptococci | 7 | 16 | 100 | 88 | 88 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

cases of poliomyelitis. Specific effects were especially striking after inoculation of filtrates and suspensions of heat-killed streptococci, when ultimate dosage could be controlled better than by inoculating the living streptococci.

The mortality rate varied greatly after inoculation of living streptococci, being highest in animals that received the streptococci from patients having influenza (90 per cent) and in those that received streptococci from air of rooms of persons having epidemic hiccup (87 per cent) or poliomyelitis (86 per cent); intermediate after inoculation of streptococci from throats of patients suffering from epidemic hiccup (58 per cent) or postoperative hiccup (53 per cent), or of well persons remote from epidemics (42 per cent); and lowest (33 per cent) after inoculation of streptococci from milk supplies.

Lesions of the diaphragm, consisting of localized hemorrhages, occurred only in animals that had violent spasms after inoculation of streptococci isolated from persons having persistent hiccup.

Lesions of the lungs, consisting of hemorrhagic edema and bronchopneumonia, occurred most often and, with one exception, were most conspicuous after inoculation of the streptococcus isolated from persons having persistent hiccup or from milk and air in relation to persistent hiccup and influenza. In accord with the frequent occurrence of gastric distress in patients, lesions of the stomach were common and consisted of numerous, small, superficial hemorrhages in the mucous membrane apparently due to violent spasms, and of early, often almost immediate, post-mortem digestion of the wall of the stomach.

The incidence of isolation of streptococci from the brain, the site of inoculation, was relatively high in each group of cases, whereas the incidence of their isolation from blood of animals that died was very low (0 to 9 per cent), with the exception of those inoculated with streptococci from the nasopharynges of persons having acute influenza (67 per cent).

The spasm-producing type of streptococcus was isolated repeatedly from some patients during acute attacks of hiccup but not after recovery. Its presence was demonstrated in persons during recurring attacks over periods as long as four years, in atria of infection in persons having mild but continuous symptoms for as long as two years, and in several protracted cases in which the attending physician considered the spasms of functional origin. In some cases it was isolated from nasopharynx, infected teeth, tonsils, prostatic secretion, urine, stool, or pus from appendiceal abscesses and infected gall bladders.

### MICROSCOPIC LESIONS OF THE BRAIN AND SPINAL CORD OF EXPERIMENTAL ANIMALS

Sections of the brain and spinal cord were made and examined for lesions in altogether 39 animals that died from inocula or that were anesthetized after inoculation. The duration of the experiment in these animals was one to two days in 14; three to five days in 15, and six days or longer in 10. Suspensions in gelatin-Locke's solution of nasopharyngeal swabbings and other material directly from patients were inoculated in 12 animals, cultures of living streptococci in 21, dead streptococci in two, and filtrates in four.

The lesions found in animals that died within one to two days after inoculation consisted chiefly of a relatively mild, but never grossly suppurative, polymorphonuclear meningitis, usually most pronounced over the anterior aspect

of the medulla. Perivascular, leucocytic infiltration, degeneration, edema and localized hemorrhages in sulci, choroid plexus, and anterior aspect of the me-

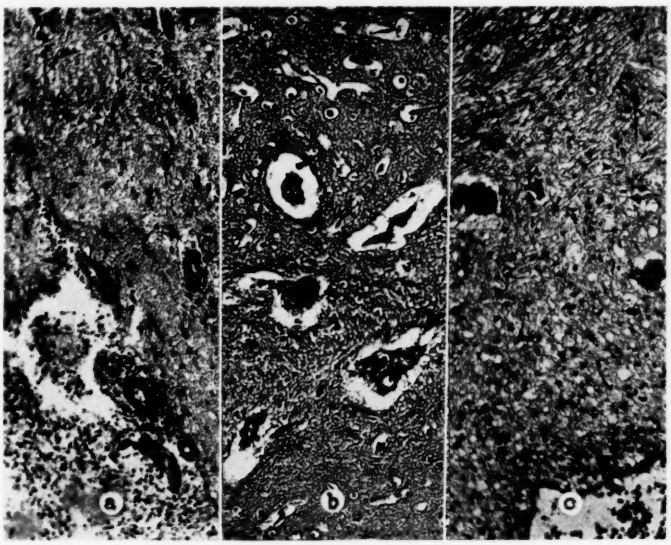

Fig. 2.—Lesions in rabbits in which spasms of the diaphragm developed after intracerebral inoculation of living streptococci isolated from the nasopharynx of a patient having epidemic hiccup: *a*, in the medulla; *b*, in the brain of a rabbit five days after inoculation; *c*, in the medulla of another rabbit three days after inoculation of the same culture (hematoxylin and eosin, ×110).

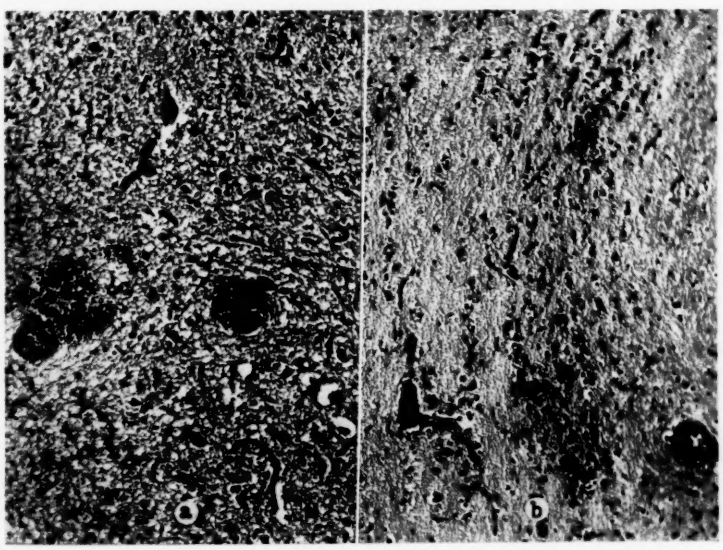

Fig. 3.—Lesions in animals in which spasms of the diaphragm had developed after intracerebral inoculation of living streptococci from the nasopharynges of patients having postoperative hiccup: *a*, in the medulla of a guinea pig six days after inoculation; *b*, in the pons of a rabbit five days after inoculation (hematoxylin and eosin, ×110).

dulla were often conspicuous two days after inoculation of material containing living streptococci, dead streptococci, and active filtrates. Following this, there was a rapid transition from polymorphonuclear cells to lymphocytes,

especially surrounding blood vessels in the pons, floor of the fourth ventricle, medulla, subcortical region, and choroid plexus. This transition in type of infiltrating cells occurred earlier within the parenchyma than in the meninges (Fig. 2). After five or six days typical lymphocytic infiltration, degeneration of ganglion cells (Fig. 3), circumscribed regions of glial infiltration, and other lesions of encephalitis were found. In no instance were the lesions characteristic of spontaneous encephalitis encountered. Necrosis associated with leucocytic and lymphocytic infiltration at the point of injection in the right frontal lobe occurred commonly, but abscess formation was present only occasionally. The lesions remote from the point of injection were essentially alike on both sides. The streptococci were readily demonstrable, especially as diplococci, in the lesions of animals that died soon after inoculation. As lymphocytic infiltration became marked, their demonstration became difficult and often impossible (Fig. 4).

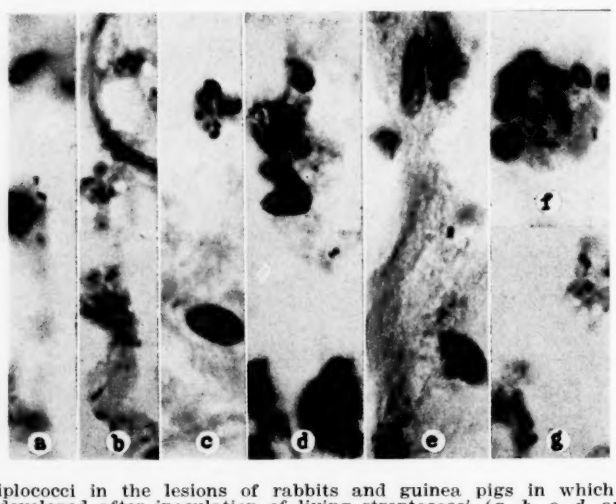


Fig. 4.—Diplococci in the lesions of rabbits and guinea pigs in which spasms of the diaphragm had developed after inoculation of living streptococci (*a*, *b*, *c*, *d*, and *f*) and heat-killed streptococci (*e* and *g*) isolated in studies of epidemic and postoperative hiccup (modified Gram stain, ×1,000).

## SERUM TREATMENT

Serum treatment was given only after the usual methods, such as administration of carbon dioxide, gastric lavage, and sedatives, to control the spasms had proved of no avail. The whole, unconcentrated encephalitis antistreptococcic serum was used at first, whereas later the refined serum of similar antibody content, consisting of solutions of one part of the euglobulin and two parts of the pseudoglobulin, was used. After determining that the patient was not allergic to horse serum, and after desensitization if he was found sensitive, 10 c.c. of the serum were injected deeply into the muscles of the buttock twice daily until the hiccup had disappeared, and usually one additional injection was given after that. The concentrated encephalitis antiserum and the euglobulin solution for the cutaneous test, first prepared by me, are now being made available for study by the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.

Records of results from the use of encephalitis antistreptococcic serum in treatment have been obtained in altogether 90 cases of persistent hiccup. Fifty-four of these were considered as cases of epidemic persistent hiccup and 36 occurred postoperatively. The results from the use of the serum were so similar in the two groups of cases that they will be considered as one group.

The ages of the patients ranged from 7 to 82 years. The great majority were more than 50 years of age. In all but two postoperative cases, and all but six of the nonoperative cases the patients were men, a proportion of approximately ten men to one woman. The duration of the disease at the time of the first serum treatment was from one to twenty-one days in acute cases, as long as two years in persons having continuous hiccup, and four years in those having recurring attacks. The amount of serum given ranged from 10 to 110 c.c., usually 20 to 40 c.c.

Detailed records regarding noteworthy diminution of severity and number of spasms and cessation of hiccup in relation to the number of injections of serum were available in 60 cases. Reduction of severity and number of spasms was noted after the first injection of serum in 20 cases, after two injections in 21 cases, and after three or more injections in 12, whereas in seven cases no apparent benefit followed soon after injections of serum. Cessation of spasms occurred after the first injection in 14 cases, often within several hours; after two injections in 16 cases, after three injections in nine, and after four injections in 10 cases. In the remaining seven cases in which the patients survived, the hiccup disappeared after from seven to ten days during the period of sensitization. In four postoperative cases no apparent beneficial effects were noted after the administration of the serum. Two of these patients died: one, a man 78 years of age, from cardiac failure following suprapubic prostatectomy in the presence of a badly infected bladder; the other, a man 72 years of age, from peritonitis following strangulated hernia. In the other two, hiccup occurred after prostatectomy, continued for two weeks, and then gradually disappeared.

Immediate serum reactions did not occur in any case. In 15 there were late serum reactions and of these only five were severe.

In the 36 postoperative cases 11 patients had had prostatectomy; two, cystoscopy; five, appendectomy with appendiceal abscess; three, colostomy; seven, cholecystectomy; two, herniotomy; one, gastrectomy; two, rectal resections for carcinoma; one, nephrotomy; one, septic hip; and one, tonsillectomy. There was great variation in the effects of the serum after its injection. The sudden permanent cessation from one or two injections, after every other known means had failed to give relief, often in patients severely exhausted, was so convincing as to leave little doubt of the curative action of the serum. The earlier the serum was given, the more prompt was the relief. The cessation of hiccup after administration of the serum was not coincidental because usually more injections were necessary late in the course of attacks than when given early. Prompt relief after administration of the serum was often associated with striking diminution in reactivity of the skin on reinjection of the encephalitis euglobulin and a negative precipitation reaction with the patient's

serum. This was true of both the nonoperative and operative cases. These observations are in accord with the results of treatment reported by Mayo, assisted by Rozendaal, who stated:

"That there is a relationship between the organisms of epidemic encephalitis and of epidemic hiccup is fortunate, because the encephalitis antibody globulin solution as prepared by Rosenow can be applied in treatment. Such treatment was given in those of our cases in which persistent hiccup was present, and we were able to recover the neurogenic streptococcus from the throat and reproduce spasms in animals. The effect of the serum was dramatic in many instances, all symptoms of hiccup disappearing within a few hours."

Most of the patients whom I saw had badly infected teeth or other evident foci of infection. There was definite correlation between focal and metastatic infection and the response to serum treatment. In general, the worse the infective process, the longer were injections of serum necessary to control the hiccup. This was especially true in cases in which there was surgical drainage after operative treatment of suppurative appendicitis, cholecystitis, and prostatitis associated with cystitis. In several cases in which the diagnosis was functional hysterical hiccup, the spasm-producing streptococcus was isolated from the nasopharynx and prompt relief followed administration of the serum.

As in epidemic encephalitis and poliomyelitis, there has been found a much higher incidence of cases of persistent hiccup—a mild form of myoclonic encephalitis—among men than among women, indicating an inherent susceptibility of men to the inciting agents of these diseases.

## COMMENTS AND CONCLUSIONS

The results of previous studies have been corroborated and extended. The isolation of the spasm-producing type of streptococcus from the nasopharynx, milk supplies, and outdoor air during epidemic prevalence of hiccup and certain respiratory infections and from the air of rooms occupied by persons having persistent hiccup, and the absence of this type of streptococcus remote from hiccup, indicate that epidemic hiccup and postoperative hiccup occur in increased incidence when streptococci normally present in throats of persons and in nature generally acquire spasm-producing properties or virulence.

Persistent epidemic hiccup and persistent postoperative hiccup are considered as forms of mild myoclonic encephalitis. The inciting agent is a streptococcus *(Streptococcus singultus)* closely related to the streptococcus isolated in studies of epidemic encephalitis and the antistreptococcic serum for epidemic encephalitis is curative in persistent hiccup.

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# CIRCULATING TIME IN THE HUMAN BEING AND IN THE DOG AS AFFECTED BY FASTING AND BY MEALS*

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OUR laboratory has been interested in the use of determinations of circulating time in the state of shock.[1] We were interested, therefore, in the normal variations of circulating time during fasting and following the ingestion of various foods. Our results obtained differ from those of another group.[7, 8]

Various reports in the literature indicate that there is a considerable change in circulation during digestion in man. According to Grollman[2] and others, a significant increase in cardiac output occurs in man after ingestion of food and even of water. Booth and associates[3] reported an elevation of skin temperature in normal subjects following a meat meal. Herrick and associates[4] did not obtain an increase of surface temperature in dogs following ingestion of foods.

In 1934 Herrick and associates[4] reported a notable increase in blood flow in the femoral, carotid, and mesenteric arteries, and in the external jugular vein of the dog following ingestion of various foods. The time of the onset and the duration of the increased flow were influenced by the character of the food taken, but the magnitude of increase was relatively the same regardless of the type of food. These results were confirmed in the human being by Burton and Murlin.[5] Abramson and Fierst,[6] working on human subjects, did not find significant changes in the rate of peripheral blood flow following a carbohydrate meal, but with a protein meal the rate of blood flow increased one and one-half to three hours after ingestion, first in the hand, and later in the forearm and leg.

In 1934 Sheard and co-workers[7] reported experiments in which the circulating time of the blood of dogs was reduced 20 to 35 per cent during digestion of a mixed meal. The lowered circulating time coincided with increases in blood flow measured simultaneously. The method used by these workers consisted in the injection of radium C into the jugular vein and the measurement

*From the Department of Gastro-Intestinal Research, Michael Reese Hospital, Chicago.
Supported by the Emanuel Friend Fund.
Received for publication, June 29, 1942.

of the interval of time between the injection and the arrival of the radium below an ionization chamber located over the femoral artery. In 1936 they confirmed these results.[8]

## METHODS

In the work reported here we have used the sodium cyanide method for the determination of circulating time, as first described by Loevenhart and associates[9] and as applied by Robb and Weiss to the human being.[10] The cyanide method consists of the intravenous injection of a small quantity of sodium cyanide and the measurement of the time elapsing between the injection and the first response of the chemoreceptors of the carotid sinus which stimulate respiration. In the dog 1 mg. of sodium cyanide dissolved in 0.5 c.c. of saline was injected into the saphenous vein of the hind leg, and in man 8 mg. of sodium cyanide in ⅓ c.c. of saline solution was injected into the antecubital vein. The injection was given rapidly, and two stop watches were started at the time of injection. At the height of the first deep gasp one of the stop watches was arrested. The time obtained was considered to be the circulating time of the blood between the peripheral vein and the carotid sinus. The reaction consisted of a series of rapid and deep respirations. Two stop watches were used because an occasional deep breath not followed by a series of rapid and deep respirations might yield a false end point, which could be corrected by the use of the second stop watch when the series of rapid and deep respirations began.

The human subjects and the animals were starved for at least twelve hours. The experiments on the dogs were performed on two healthy animals and those on the human subjects, on workers in the laboratory, and on such patients of the wards who had undergone simple operations and were ready to be dismissed, and on such patients in whom the circulatory system did not seem to be affected by the disease. The ages of the subjects varied between 16 and 79 years, with an average of 49 years. Most of the human subjects were lying in bed during the experiments, and a few were up and around. The dogs were in a stall during the entire experiment.

A meal was administered after one or more control determinations of the circulating time had been performed, and determinations were repeated at various intervals following ingestion of the meal. One group of subjects was followed over a prolonged period of time without administration of food. The meals administered consisted of protein foods (steaks), or of a regular full hospital diet, or of carbohydrate and fat foods. The amounts eaten were about those of a good average luncheon and varied somewhat according to the appetite of the subject. The dogs were fed one pound of a commercial dog food containing meat, oats, and vegetable, mixed with water.

Twenty-nine experiments were performed on 21 different subjects: 17 males and 4 females. Twelve experiments were performed on 12 different fasting subjects and are recorded on the upper part of Chart 1. Six experiments were performed on two dogs.

## RESULTS

A. In Human Subjects.—Fasting circulating times varied between 12 and 22 seconds, with an average of 17.2 seconds (34 tests). Controls before meals varied between 12 and 29 seconds, with an average of 17.6 seconds (31 tests).

Circulating times following all meals varied between 10 and 26.6 seconds, with an average of 17.5 seconds (48 tests). Analysis of the data did not reveal significant differences in the changes of circulating time following the various meals. In order to illustrate the wide variations of circulating time during fasting and following meals, we have expressed all data as per cent changes from the first determination in the fasting subjects and from the last determination before the meal in the postprandial subjects. We have chosen this method of presentation of our data because the variations observed during the fasting and the postprandial states seemed to be due to cyclic changes.

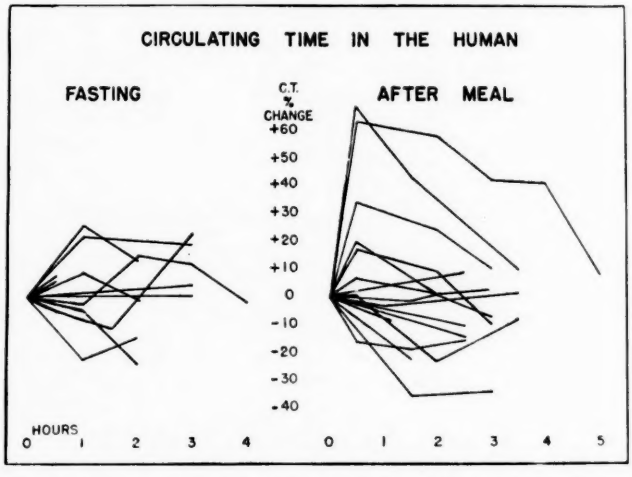


Chart 1.—Circulating time in the *fasting* human subject in per cent changes from first determination. Circulating time *after meal* in per cent changes from last determination before meal.

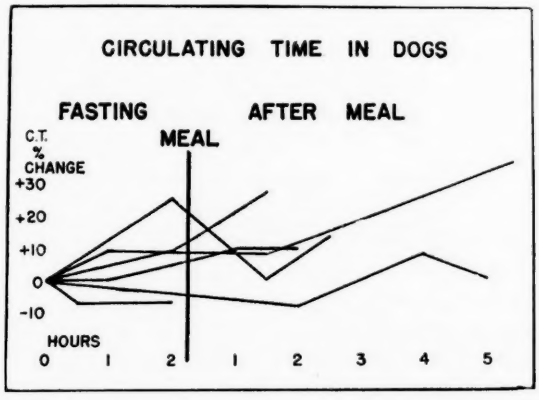


Chart 2.—All circulating times in per cent changes from first determination made.

In most of the fasting subjects variations between −25 and +25 per cent from the control reading were obtained, while in only a few of the fasting subjects circulating time did not vary appreciably during the periods of observation of thirty minutes to four hours.

Seventeen experiments following ingestion of food are recorded on the right side of Chart 1. Circulating time varied between a diminution of 36 per cent and an increase of 67 per cent of the control values. The spread of the

data is greater than that seen during fasting, but this is due to a greater variation in circulating time in only 2 of these 17 experiments.

B. In the Dog.—Values of circulating time before the meal varied between eight and fourteen seconds, with an average of twelve seconds (12 tests), and after the meal they varied between eight and fifteen seconds, with an average of 12.2 seconds (11 tests). Chart 2 illustrates that essentially the same results were obtained in the dog as in the human being. The circulating time was not constant during periods of two hours of fasting and did not show uniform changes in any direction during periods of one and one-half to five and one-half hours following ingestion of the protein meal.

## DISCUSSION

Our results indicate that circulating time varies considerably during fasting as well as following ingestion of various foods. These variations were not uniform in direction, and prolongation as well as shortening of circulating time might occur. From the distribution (spread) of data, as well as from the algebraical averages we assume, therefore, that the changes of circulating time following a meal are not any more significant than are those during fasting. The variations of the individual curves during fasting as well as following meals may be due to cyclic variations in circulating time, rather than to specific causes, such as anxiety, the postabsorptive state, etc. If it can be proved that this is due to cyclic variations in circulating time, it will make the simple use of this method uncertain for a number of clinical determinations. The use of the method is not without value in selected conditions, however, as, for example, in the state of shock, where we have described changes far beyond those observed in normal dogs.[1]

We cannot explain the difference between our results and those of the Mayo group.[7, 8] Of course, the two methods are entirely different, and it is possible that they are affected differently during the absorptive state. The sensitivity of the chemoreceptors of the carotid sinus is so great that differences in their response to cyanide constitute only a small fraction of the total circulating time,[1] and we feel that a meal could not affect these receptors so much as to change the total circulating time to any extent. There may be other factors at work, however, such as changes of the circulating volume or of the hematocrit-plasma ratio during the absorptive state, or differences in the carriers (red blood cells or plasma) of the different agents employed, which may affect both methods differently. Besides, it was not apparent from the report of the Mayo group whether prolonged control periods during starvation had been instituted and had been compared with the variations of circulating time following meals.[7] In another paper, however, this group reported considerable variations of the circulating times in individual fasting dogs.[11]

The reported constant differences between the cyanide method and arm-to-tongue methods[12] would not seem to affect our findings.

We feel that our results are also of importance because the type of method employed in our study for determination of circulating time belongs to those in common use.

SUMMARY

The circulating time was determined in a group of human subjects and in dogs. The sodium cyanide method of Loevenhart and associates was employed. During the fasting condition considerable variations of circulating time were found in the human being and in the dog, and following meals the same or somewhat greater variations in circulating time were obtained. Our results indicate no significant or uniform changes in circulating time in any one direction in either the fasting or the postprandial state. The nature of the meal did not seem to determine changes in circulating time. The significance of our findings and the discrepancy of our results from those of other workers are discussed.

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# RECURRENT ATTACKS OF PNEUMOCOCCIC PNEUMONIA TREATED WITH SULFONAMIDES*

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RECURRENCE is more commonly seen in pneumococcic pneumonia than in any other acute infectious disease.[1] Likewise, the attack rate of pneumococcic pneumonia is higher in those persons who have had previous attacks of the disease.[2] The reported incidence of one or more previous attacks varies greatly throughout the world, although most American authorities have noted recurrences in approximately 15 per cent of cases. Furthermore, it is the opinion of many writers[3, 4] that the number of previous attacks of pneumococcic pneumonia does not influence the prognosis of the disease. Within the past four years the treatment of pneumococcic pneumonia has been revolutionized as a result of the advent of the sulfonamide compounds. It is too early to predict whether the widespread use of these drugs will have any effect upon the above-mentioned facts concerning recurrent pneumococcic pneumonia, since it will be necessary to accumulate extensive data from different sources over a period of years before an evaluation can be attempted. However, of equal interest is whether these chemotherapeutic agents are as effective when employed for repeated attacks of pneumococcic pneumonia and whether the patients tolerate the sulfonamide drugs as well during subsequent administrations. The purpose of this study is to present the comparative therapeutic effectiveness and toxicity of the sulfonamides in recurrent pneumococcic pneumonia.

During the past three years we have observed in a series of 1,400 adult pneumonias 24 patients who received sulfapyridine, sulfathiazole, or sulfadiazine on two or more occasions for pneumococcic pneumonia. In every instance the patient was discharged from the hospital fully recovered from pneumonia; and, in order to eliminate possible cases of relapse, we have excluded those readmitted within four weeks following discharge. The diagnosis of pneumonia was established in every case, and a pneumococcus was recovered from the sputum or blood in all patients, although a specific pneumococcus type was not always obtained.

As indicated in Table I, there was a total of 49 hospital admissions in this group of 24 patients. The time interval between admissions ranged from four to ninety-two weeks, with an average of thirty-seven weeks. The patients' ages varied from 14 to 74 years, with 58.3 per cent of the cases being over 40 years of age. Typable pneumococci were found in the sputum or blood stream in 35

*From the Committee for the Study of Pneumonia, Philadelphia General Hospital. Aided by a grant from the American Philosophical Society.
Received for publication, July 8, 1942.



TABLE I
SIGNIFICANT DATA ON 24 CASES OF RECURRENT PNEUMOCOCCIC PNEUMONIA TREATED WITH SULFONAMIDES

TABLE I

SIGNIFICANT DATA ON 24 CASES OF RECURRENT PNEUMOCOCCIC PNEUMONIA TREATED WITH SULFONAMIDES

| | FIRST ATTACK | | | | | | INTERVAL BETWEEN ATTACKS (WEEKS) | SUBSEQUENT ATTACKS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NO. | AGE YR. | TYPE | LOBES INVOLVED | TREATMENT | RESULTS | TOXIC EFFECTS | | TYPE | LOBES INVOLVED | TREATMENT | RESULTS | TOXIC EFFECTS |
| 1 | 68 | VIII | RM, RL | S-P* | Good | Vomiting | 32 | III | RL | S-P | Good | Vomiting |
| 2 | 57 | VI | RL | S-P | Good | | 13 | V | RL | S-P | Good | |
| 3 | 37 | I | LL | S-P Serum | Good | Vomiting | 4 | – | RL, LL | S-P | Good | Vomiting |
| 4 | 74 | III | RL, LL | S-P | Good | Vomiting, microhematuria | 20 | III | RL | S-P | Good | Vomiting, microhematuria |
| 4a† | | | | | | | 8 | III[b] | RL, LL | S-P | Died | Vomiting, microhematuria |
| 5 | 21 | – | RU, RM | S-P | Good | | 4 | XIV | RU | S-P | Good | |
| 6 | 42 | VII | LL | S-P | Good | | 37 | VIII | RL | S-P | Good | |
| 7 | 14 | IV | LL | S-P | Good | | 56 | – | LL | S-P | Good | |
| 8 | 40 | – | RL | S-P | Fair | | 33 | – | RL, LL | S-P | Fair | |
| 9 | 68 | VII | RL | S-P | Good | Vomiting | 62 | VII | RL | S-T | Good | Vomiting, microhematuria |
| 10 | 66 | V[b] | LU | S-P | Fair | | 22 | I | RM | S-T | Good | |
| 11 | 49 | VIII | LL | S-P Serum | Fair | | 42 | – | LL | S-T | Good | |
| 12 | 32 | III[b] | RL, LL | S-P | Good | Vomiting | 58 | VIII | RL, RM | S-T | Good | |
| 13 | 27 | III | LU | S-P | Good | Vomiting | 40 | VII | RL | S-T | Good | |
| 14 | 25 | VII | RL, LL | S-P Serum | Fair | Vomiting | 26 | – | LL | S-T | Good | |
| 15 | 55 | – | LL | S-P | Good | Vomiting | 18 | – | LL | S-T | Good | Vomiting |
| 16 | 37 | XXIV[b] | LL | S-P | Good | | 92 | XIX | LU | S-D | Good | |
| 17 | 60 | – | LU | S-P | Good | Microhematuria | 84 | XXIX | LU, LL | S-D | Good | |
| 18 | 27 | – | RM | S-T | Good | | 4 | XIV | LL | S-T | Good | |
| 19 | 53 | VIII | LU | S-T | Fair | Vomiting | 42 | VIII | RL | S-T | Fair | |
| 20 | 66 | V[b] | LU | S-T | Fair | Microhematuria | 22 | I | RM | S-P | Good | |
| 21 | 44 | III | LL | S-T | Good | | 39 | III | RL, LL | S-P Serum | Died | Vomiting |
| 22 | 48 | VII | LL | S-T | Good | | 52 | III | RL | S-D | Good | |
| 23 | 26 | – | LU | S-T | Good | | 49 | I[b] | RL | S-D | Good | |
| 24 | 57 | – | RL | S-T | Fair | Vomiting, microhematuria | 53 | – | LL | S-D | Fair | |

*S-P = Sulfapyridine.
S-T = Sulfathiazole.
S-D = Sulfadiazine.
†4a = Third admission.
b = Positive blood culture.

instances. The same pneumococcus type was the causative agent for subsequent attacks in only 4 patients. Positive blood cultures were obtained in 4 patients on the initial admission and in 2 patients on subsequent admission to the hospital. In 14 patients the same lobe of the lung was involved during each attack of pneumonia, and in 9 of this subgroup an additional lobe was involved. There was no appreciable difference in the total number of lobes involved during the initial and subsequent attacks.

Sulfapyridine was employed in 17 patients during their first admission, and of this number 8 received the same drug on nine subsequent admissions. The remainder of this subgroup received sulfathiazole (7 patients) or sulfadiazine (2 patients). Seven patients were given sulfathiazole on their first admission, and of these 2 received sulfapyridine, 2 sulfathiazole, and 3 sulfadiazine on other admissions. Good therapeutic response to treatment with these drugs was determined by a critical fall in temperature within forty-eight hours, a return to normal temperature within forty-eight to seventy-two hours, and by the findings on physical examination. On this basis we have designated the results of treatment as good, fair, or died. There were 2 deaths (8.0 per cent mortality) in this group of 25 subsequent admissions, both occurring in patients with type III infection. This mortality rate compares favorably to that in a larger series of cases of pneumococcic pneumonia treated with sulfonamides reported from this hospital.[5] Of the 22 patients surviving both attacks of the disease, the results of treatment were considered good in 15 on their first admission and in 20 during subsequent courses of therapy with these drugs. This difference in therapeutic response on the different admissions is of no statistical significance.

The incidence of severe toxic reactions due to the drugs is very small in this series. Vomiting and microscopic hematuria constituted the only toxic manifestations worthy of mention. In comparing these toxic effects it should be pointed out that the selection of drug undoubtedly influenced the incidence of toxicity in these cases. However, it is of interest to note that none of the more serious toxic reactions developed on subsequent use of the same drug or of other members of this group of drugs. Not included in this series of cases is a patient recently treated by one of us (H. F. F.) for pneumonia with sulfadiazine who showed no apparent ill effects from the drug; this patient had on a previous occasion developed a severe skin reaction as a result of sulfathiazole medication.

In view of the data presented in this report it would appear that the use of the sulfonamides in a previous attack of pneumococcic pneumonia does not alter the response to the same drug or to similar drugs when used in subsequent attacks of the disease. Also, the incidence of toxic reactions is no greater when these drugs are employed for repeated attacks of pneumococcic pneumonia. Further study of similar data on a larger series of cases receiving the same drug for two or more attacks of pneumococcic pneumonia is desirable.

## SUMMARY

1. Twenty-four adult patients have been given sulfonamide therapy on two or more occasions for recurrent pneumococcic pneumonia.

2. The drugs seemed just as effective when administered for subsequent attacks of the disease as during their initial use.

3. There is no evidence to suggest that the repeated use of the sulfonamides for recurrent pneumococcic pneumonia increases the incidence or severity of drug toxicity.

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# ERYTHROCYTE SEDIMENTATION RATE DETERMINATIONS ON NORMAL YOUTHS

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THE erythrocyte sedimentation rate is an accepted laboratory measure of pathologic activity, and the range of normal values for the various techniques has been determined by numerous investigators over the country. Rourke and Ernstene found the normal range of "corrected sedimentation index" to be 0.08 to 0.35 mm. per minute, based on the results of 92 measurements done on 45 different healthy persons.

In order to ascertain what is the range of normal in this community, determinations were done on 100 normal youths, all students at the junior high school and high school here. A careful history and physical examination were done on each subject, and only those found to be in good health were included in the series. Any history of recent infection, common cold, tonsillitis, dental infection, sinusitis, etc., excluded the potential subject. Of the 100 healthy youths in the series, 60 were males and 40 were females. Their ages ranged from 11 to 17 years.

The temperature of each subject was recorded, and all blood specimens were obtained at least two hours after the previous meal. Extreme care was taken in order that blood be withdrawn from the vein with as little stasis as possible. Any apprehension or excitement on the part of the subject at the time the specimen was withdrawn was recorded. The date on which the last menstrual flow ceased was noted for each female subject.

The Rourke-Ernstene corrected sedimentation technique was used exclusively. Each determination was made within two hours after the blood speci-

Received for publication, July 6, 1942.

men was obtained. Heparin was used as the anticoagulant. Every sedimentation tube was handled with a minimum amount of manipulation. Precautions were taken to see that the sedimentation tubes were in absolute vertical position. The temperature and humidity of the laboratories and the barometric pressure were recorded. Sixty-two determinations were done in the St. Francis Hospital laboratory, which is not air-conditioned, and the remaining 38 tests were done in an air-conditioned laboratory.

The corrected sedimentation rates of the 100 normal youths ranged from 0.09 mm. per minute to 2.0 mm. per minute. The mean rate was 0.46 mm. per minute. Seventy-five per cent of all the determinations were below 0.65 mm. per minute. Rourke and Ernstene[1] placed the value of 0.35 mm. per minute as the upper limit of normal. Yet 53 per cent of all the readings in this series fell in the range between 0.35 and 0.65 mm. per minute. The mean rate for girls was 0.47 mm. per minute, and for boys it was 0.46 mm. per minute.

We could not determine the reason that the sedimentation phenomenon should be more rapid in a subtropical climate. Numerous investigators have shown that low temperatures retard and high temperatures accelerate the rate of sedimentation of erythrocytes. The barometric pressure varied from 760 mm. to 770 mm.

Forty-four volumes per cent was found to be the mean hematocrit value for both males and females in our series. Rourke and Ernstene have set 45 volumes per cent as the mean value for correcting the sedimentation rate. Forty-nine volumes per cent was the highest value for females in the series, while 11 of 60 males had hematocrit values of 50 to 52 volumes per cent.

## CONCLUSIONS

1. The erythrocyte sedimentation rates of 100 normal Miami Beach youths were determined according to the Rourke-Ernstene technique.

2. The rates of 75 per cent of the series ranged from 0.09 to 0.65 mm. per minute, and 53 per cent of the series ranged from 0.35 to 0.65 mm. per minute.

3. We propose that the upper limit of normal for the Rourke-Ernstene corrected sedimentation index for South Florida be extended to 0.65 mm. fall per minute.

4. Since it has been proved by other workers that the various methods for determining erythrocyte sedimentation rate are reasonably the same, we suggest that the range of normal for other techniques also be extended for this locality.

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# PAPILLARY LYMPHOID CYSTADENOMA*

## Report of Four Cases

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AMONG tumors of the salivary glands one of the rarest is papillary lymphoid cystadenoma. Other terms by which it has been described include papillary adenolymphoma, papillary cystadenoma lymph glands, papillary cystadenoma lymphomatosum, branchiogenic cystadenolymphoma, branchiogenic adenoma, branchioma, and onkocytoma. Papillary lymphoid cystadenoma is probably the most descriptive name for the tumor.

The tumors were first described by Albrecht and Arzt (1910), Glass (1912), Ssobolew (1912), and Feldman (1916). In 1935 Carmichael, Davie, and Stewart reviewed the literature and found 26 cases reported up to that time in twenty-one papers. They described 8 new cases. Subsequently, five more cases appeared in the American literature,[6, 8, 9, 11] and seven in foreign language publications.[10-16]

The genesis of these tumors has been variously ascribed to heterotopic salivary gland tissue in lymph nodes,[3, 4] heterotopic pharyngeal epithelium,[17] and to branchiogenic rests.[18] The great majority of the tumors are first noticed in the fifth or sixth decade, and they are found only in close relation to the parotid and submaxillary glands and at the angle of the jaw. No case has been noted in association with the sublingual glands. They are six or seven times more common in males than in females. Only two of the cases have been stated to be malignant. One tumor reported by Oden[6] showed enough active proliferation and loss of polarity of the cells to be possibly a transition stage between benignity and malignancy. Only one case was reported in which there was a local recurrence and this appeared in three years.

In the surgical biopsies of Hines Hospital during the past two years five cases of papillary lymphoid cystadenoma were found. One of these has been reported by Hines.[11] All of them were benign.

### CASE REPORTS

Case 1.—The patient, W. S. D., was a white male, aged 51 years. One and a half years before admission to the hospital he noticed in the preauricular area on the right a small lump which had grown progressively larger. On physical examination this mass was small and movable.

At operation the tumor was enucleated from under the capsule of the parotid gland. Another smaller nodule was then found adjacent to the one previously removed, and it was also removed. The tumors measured 2 by 1 by 1 cm. and 1.5 by 1 by 0.5 cm.

Case 2.—Three and a half years before admission to the hospital a white male, A. J. K., aged 49 years, observed in the right parotid region a growth which gradually

*From the Clinical Laboratory Center, Veterans' Administration, Hines, Ill.
Published under R & P 6227.
Received for publication, July 13, 1942.

increased in size. No discomfort or pain was associated with the lesion. He was operated on at another hospital, and a partial excision was done. The biopsy diagnosis was made there of adenoma of the parotid with malignant change. Upon admission to the hospital for further therapy one month later a soft 2 cm. mass was found in the right parotid area. The tumor was raised 1.5 cm. above the skin. Sialogram revealed filling of the ducts of the parotid gland which was compressed by the external tumor.

At operation a nodule was found in the substance of the parotid gland. The tumor was completely encapsulated and easily enucleated. It measured 4.5 by 2.5 cm.

CASE 3.—Patient, R. C. B., a white male, aged 48 years, noticed a small lump behind the left ear, five months before admission to the hospital. The mass was about the size of a pecan and had grown steadily larger. The patient experienced no pain and no increase in salivation.

Physical examination revealed a 5 by 4 cm. mass in the left parotid region. The overlying tissues were freely movable, but the tumor appeared to be somewhat fixed to the underlying structures. No adenopathy was present.

At operation the tumor was found to be within the parotid gland substance but well encapsulated and easily enucleated. It measured 5.3 by 3.5 by 2 cm., felt cystic, and had small satellite nodules at the periphery.

CASE 4.—The patient, G. F. J., a white male, aged 46 years, noticed a mass behind the angle of the left jaw three years before admission to the hospital. This became progressively larger. The nodule was asymptomatic.

Physical examination revealed a tumor the size of a walnut behind the angle of the left jaw. The skin was freely movable over it. The mass was slightly movable but was apparently attached to, and was part of, the parotid gland underneath.

At operation this mass was found in the lower pole of the parotid gland. It was encapsulated and was easily enucleated. It measured 4 by 3 by 2 cm.

The preoperative duration in our cases was eighteen months, forty-two months, five months, and thirty-six months, respectively, and averaged twenty-five months. The tumors were in the substance of the parotid gland in three cases and just under the capsule in the first case. None of the masses were painful or tender. The tumor was not attached to the skin in any of the cases. The right and left parotid areas were equally affected in frequency. The preoperative diagnosis in three cases was mixed tumor of the parotid gland. In our second case the preoperative diagnosis was adenoma of the parotid with malignant change. All the tumors were well encapsulated and easily removed.

The capsule of the tumors was thin, fibrous, and had small vessels on its surface. The color varied from a reddish brown to a reddish gray. There was a small nodular character to the surface. The tumors were cut with ease and the sectioned surface was finely granular and showed small cysts filled with serous or colloid material which upon formalin fixation became an opalescent pearly gray. The specimen in our first case, however, showed no cysts grossly.

Histologically the tumors were cystic with papillary structures. They were composed of a double layer of columnar epithelial cells which formed tubular alveoli or clefts, and cystic spaces with slender and branching papillary projections. The cells were pale and under low magnification the cell outlines were difficult to make out. Goblet cells were very prominent and numerous in the fourth case and appeared occasionally or were absent in the other cases. The basal cells were smaller, irregular, and lay close to the basement membrane. In places the cells were multilayered. The nuclei were round to oval and had a delicate chromatin network. Some of the nuclei were indented. The nuclei had

Fig. 1.—Gross appearance of papillary lymphoid cystadenoma in fourth case.

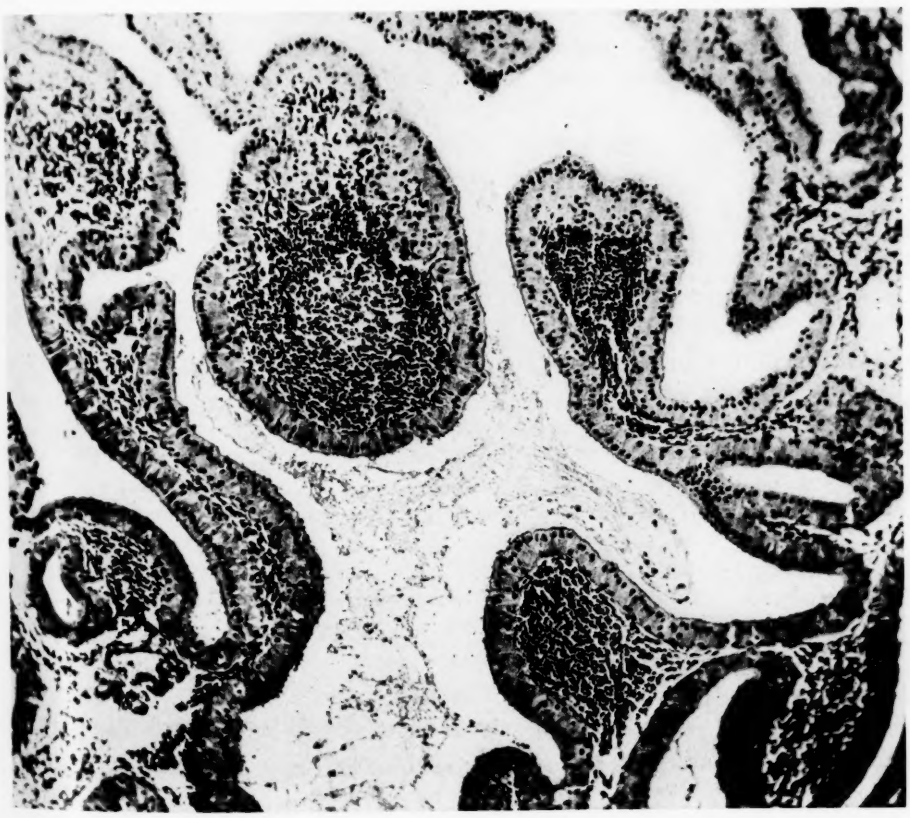

Fig. 2.—Structure of papillary lymphoid cystadenoma in fourth case, showing double layer of lining epithelium, papillary structure, and lymphoid follicles in stroma. Magnification X150.

one or two nucleoli. The epithelium rested upon a thin membrane which seemed to fuse with the delicate reticulum of the stroma in the center of the papillae. The stroma was infiltrated by many lymphocytes, with the formation of lymph follicles having large reaction centers. The cells were not ciliated. However, adherent masses of secretion gave a false appearance of cilia under low magnification.

The material in the cysts consisted of granular precipitate with desquamated epithelium, lymphocytes, phagocytes, fat, and cholesterol.

## SUMMARY

1. Of 6,239 surgical biopsies in Hines Hospital four cases of papillary lymphoid cystadenoma were encountered.

2. All four cases showed considerable consistency in clinical and gross and histological characters.

3. The clinical features were a painless and otherwise asymptomatic slow-growing mass in close association with the parotid gland. The overlying skin was movable and no ulceration occurred. The tumors were completely encapsulated and easily enucleated and they did not recur.

4. The gross appearance was characterized by a somewhat nodular capsule and a finely granular cut surface on which cysts could usually be easily seen.

5. The histology was characteristic and essentially consisted of papillary structures with a double layer of cells lining cystic spaces. The core of the papillae was made up of a reticulum infiltrated with lymphocytes forming follicles having large reaction centers.

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# THE SIGNIFICANCE OF THE ELECTROCARDIOGRAM WITH PROMINENT S WAVES IN LEADS I, II, AND III*

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ASHMAN and Hidden[1] have recently directed attention to an electrocardiographic pattern exhibiting prominent S waves in Leads I, II, and III. Eight of their 29 cases revealed no demonstrable heart disease, and 7 of their 8 cases showed no electrocardiographic changes other than the deep S waves. In all 8 cases the patients had a vertical or normally placed heart. Only one subsequent reference (Ashman and Hull[2]) to this type of electrocardiogram has appeared in the literature so far as we know.

It seemed desirable to investigate this pattern further in order to evaluate its frequency and significance. A total of 1,850 consecutive electrocardiograms from the Heart Station of Michael Reese Hospital were reviewed. Prominent S waves (defined below) in Leads I, II, and III were present in 84 of these cases (4.5 per cent). The ages represented in the 84 electrocardiograms ranged from 4 to 77 years inclusive. Fifty-nine persons were males and 25 were females.

TABLE I

CORRELATION OF CLINICAL FINDINGS WITH ELECTROCARDIOGRAMS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

| CLINICAL FINDINGS | TOTAL NO. OF CASES 84 | ELECTROCARDIOGRAM | | |
|---|---|---|---|---|
| | | NORMAL* (GROUP A) 35 | QUESTIONABLE (GROUP B) 8 | ABNORMAL (GROUP C) 41 |
| No demonstrable heart disease | 28 | 19 | 4 | 5 |
| Questionable heart disease | 10 | 3 | 1 | 6 |
| Definite heart disease | 46 | 13 | 3 | 30 |

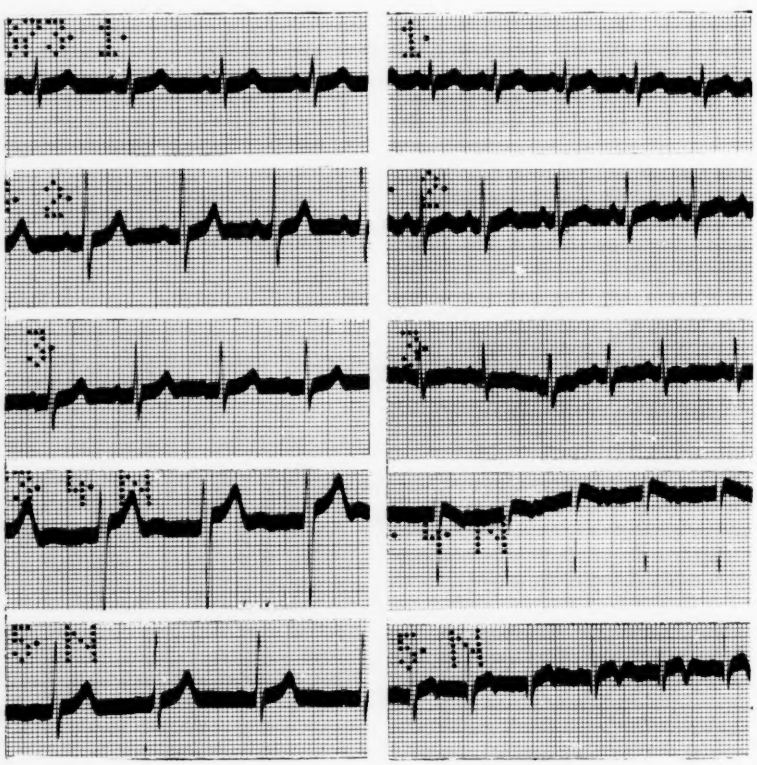
*See criteria in text.

The electrocardiograms were divisible into three groups: Group A, in which no additional deviations from the currently recognized normal electrocardiogram were found—35 cases; group B, in which the electrocardiograms showed changes which were considered questionably abnormal—8 cases; and group C, in which the electrocardiograms revealed definite abnormalities—41 cases. The relationship of this pattern to the presence of heart disease in these three groups is summarized in Table I, and their distribution by age groups is shown in Table II.

*From the Cardiovascular Department, Michael Reese Hospital, Chicago.
Aided by the A. D. Nast and Emil and Fanny Wedeles Funds for Cardiovascular Research.

Received for publication, July 13, 1942.

The electrocardiograms considered to be of the otherwise normal S type (Fig. 1) were those exhibiting (1) prominent S waves* in Leads I, II, and III, (2) absence of a prominent Q wave,† and (3) absence of other electrocardiographic changes. The criteria employed to differentiate the otherwise normal S type from the abnormal patterns were: (1) QRS of normal duration; (2) $QRS_1$ mainly upright, or small and equiphasic; (3) $QRS_2$ mainly upright; (4) $S_2$ deeper than $S_3$; (5) normal S-T-T complex in all limb leads; (6) $CF_2$ and $CF_4$ normal,‡ and (7) absence of low "voltage." In all the otherwise normal S type of records QRS in the limb leads was largest in Lead II. The 35 cases in group A fulfilled the above requirements. Those in groups B and C did not.



*A.* *B.*

Fig. 1.—Five lead electrocardiograms of the otherwise normal S type showing Leads I, II, III, $CF_2$, and $CF_4$. Record *A* is that of a normal adult and record *B* of a normal child. Note the prominent S waves in Leads I, II, and III, and the absence of other deviations from the normal. The T-wave changes in Leads $CF_2$ and $CF_4$ in *B* are normal in a child. QRS in $CF_2$ is diphasic but the upright phase is not clear in the reproduction.

Of the 13 cases in group A in which heart disease was known to be present, there were 5 patients with clinically quiescent rheumatic heart disease and suspected mitral insufficiency, 4 with angina pectoris, 2 with chronic cor pulmonale, one child with a first attack of acute rheumatic fever, and one child with postscarlatinal myocarditis.

*Final inverted phase which is 25 per cent or more of the upright phase of the QRS complex.

†Initial inverted phase which is 25 per cent or more of the upright phase of the QRS complex.

‡Three of the 84 electrocardiograms had no chest leads; two records had only one chest lead ($CF_2$); the remaining 79 had two chest leads ($CF_2$ and $CF_4$).

The abnormal electrocardiograms exhibiting S waves in the limb leads, represented by the 41 cases of group C, were divisible into 5 major patterns: (1) left ventricular preponderance, (2) right ventricular preponderance, (3) combined right and left ventricular strains,[3-5] (4) myocardial infarction, and (5) nonspecific patterns. In Table III is presented a summary of the various types of patterns encountered in the abnormal electrocardiogram of group C. The most frequent abnormalities encountered in the nonspecific patterns were S-T depressions in the limb leads, splintering and inversion of the QRS complex in Leads $CF_2$ and $CF_4$, inversion of the T wave in $CF_2$ and $CF_4$, and low "voltage."

TABLE II

AGES REPRESENTED IN THE 84 ELECTROCARDIOGRAMS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

| AGES (YR.) | 1-10 | 11-20 | 21-30 | 31-40 | 41-50 | 51-60 | 61-70 | 71-80 |
|---|---|---|---|---|---|---|---|---|
| Number of cases | 8 | 14 | 8 | 9 | 13 | 16 | 13 | 3 |
| No demonstrable heart disease | 2 | 8 | 6 | 6 | 4 | 1 | 1 | 0 |
| Questionable heart disease | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 0 |
| Definite heart disease | 5 | 5 | 1 | 1 | 8 | 13 | 10 | 3 |

TABLE III

CLINICAL STATUS AND ABNORMAL ELECTROCARDIOGRAPHIC PATTERNS EXHIBITING PROMINENT S WAVES IN LEADS I, II, AND III

| CLINICAL STATUS | TOTAL NO. OF CASES | ELECTROCARDIOGRAPHIC PATTERNS | | | | |
|---|---|---|---|---|---|---|
| | | LEFT VENTRICULAR PREPONDERANCE | RIGHT VENTRICULAR PREPONDERANCE | COMBINED RIGHT AND LEFT VENTRICULAR STRAIN | MYOCARDIAL INFARCTION | NONSPECIFIC ABNORMALITIES |
| Total | 41 | 10 | 4 | 2 | 4 | 21 |
| No demonstrable heart disease | 5 | 2 | 2 | | | 1 |
| Questionable heart disease | 6 | 1 | | | | 5 |
| Definite heart disease | 30 | 7 | 2 | 2 | 4 | 15 |

## DISCUSSION

Records with deep S waves, in Leads I, II, and III were thus encountered in 4.5 per cent (84 cases) of the 1,850 consecutive electrocardiograms reviewed; 2.2 per cent (41 cases) revealed electrocardiographic abnormalities other than the S-type pattern, indicating a definitely abnormal record; and 0.5 per cent (8 cases) showed borderline electrocardiographic variations. In 1.9 per cent (35 cases) no additional deviations from the normal were noted. This last group was labelled the otherwise normal S type. In 19 cases, or more than half of this group, this pattern occurred in patients with no demonstrable heart disease. The probabilities are that the percentage of normal hearts in this group would be much greater in a random sample of the population than in the one selected for this study, since the percentage of normal hearts included would have been much greater.

This otherwise normal S type of electrocardiogram (in which prominent S waves are present in the three limb leads without other deviations from the currently recognized normal electrocardiogram[6]) is thus encountered in ap-

proximately 2 per cent of records in electrocardiographic files. The question arises as to whether the otherwise normal S type of electrocardiogram should be regarded as a normal variant frequently observed in cardiac disease, or as an abnormal pattern often seen in the absence of evidence of heart disease. It appears to us that the assumption that it is a normal variant is preferable, since it would tend to agree with electrocardiographic studies in which normal electrocardiograms are more often seen with cardiac disease than are abnormal electrocardiograms in the absence of heart disease.[7] Nevertheless, in individual cases the occurrence even of the otherwise normal S-type record should suggest thorough investigation of the case before dismissing it as a normal variant.

## SUMMARY AND CONCLUSIONS

1. Electrocardiograms exhibiting prominent S waves (final inverted phase of the QRS complex measuring 25 per cent or more of the upright phase) in Leads I, II, and III were present in 84 cases of 1,850 consecutive electrocardiograms reviewed.

2. In 41 of these cases definite electrocardiographic abnormalities, such as left ventricular preponderance, right ventricular preponderance, combined right and left ventricular strain, myocardial infarction, and nonspecific abnormal patterns were found. In 8 others questionable abnormalities were present.

3. In 35 cases no other deviations from the normal pattern were observed and these were regarded as the otherwise normal S type of electrocardiogram. The criteria employed in this deduction are described. In 19 of these cases no demonstrable heart disease was present; in 3 the clinical findings were inconclusive; and in 13 there was clinical evidence of heart disease.

4. It is concluded that electrocardiograms exhibiting prominent S waves in Leads I, II, and III are more common in patients with evidence of heart disease than in normal persons in the population of an electrocardiographic laboratory. However, in an otherwise normal electrocardiogram the S type may be a normal variant, but before this decision is made the case should be thoroughly investigated.

We are grateful to Dr. L. N. Katz for his invaluable advice and criticism in this study.

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# CLINICAL CHEMISTRY

## ELECTROLYTE AND WATER EXCHANGE BETWEEN SKELETAL MUSCLE, "AVAILABLE (THIOCYANATE) FLUID," AND PLASMA IN THE DOG FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE*

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SHORTLY following the introduction of desoxycorticosterone acetate for clinical use in 1939, numerous studies appeared dealing with its therapeutic value in the treatment of Addison's disease and with its effect on electrolyte and water metabolism. These studies[1-6] revealed, among other things, that the administration of the substance to patients with Addison's disease or to animals in adrenal insufficiency leads to a retention of sodium and chloride, and to an increased excretion of potassium and phosphorus. The plasma sodium and chloride concentrations become elevated to normal, and the plasma and extracellular fluid volumes become increased. At the same time the plasma potassium becomes reduced, frequently to abnormally low levels. While many clinical studies showed favorable therapeutic results with the use of desoxycorticosterone acetate, a number of authors [1, 3, 7, 8] issued a warning that symptoms of overdosage may occur in certain patients, particularly those receiving high sodium and low potassium diets. Indications of an excessive dosage of the substance or of sodium chloride, or of both, may be found in the appearance of edema, rapid gain in weight, increase in blood pressure, and finally in signs of congestive heart failure. The administration of large doses of desoxycorticosterone acetate to normal dogs has not been found to lead to the above symptoms; however, such animals develop a syndrome resembling that of diabetes insipidus[9-11] and show periodic attacks of muscular weakness or paralysis.[10, 12]

The experiments included in this paper were undertaken for the following purposes: (1) to observe any changes of the plasma volume and "available (thiocyanate) fluid" from normal; and (2) to observe the electrolyte and water exchanges between skeletal muscle and plasma following the administration of the drug in large doses and after its discontinuance.

### METHODS AND CALCULATION

Adult male dogs were used in this study and were placed on a constant diet (diet suggested by Nilson[13] and supplemented by 15 Gm. of sodium chloride

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Aided by a grant from the John and Mary R. Markle Foundation.

A preliminary report upon this work was presented before the meeting of the American Society of Biological Chemists at Boston, March, 1942 (Federation Proceedings 1: 114, 1942).

Received for publication, August 7, 1942.

and 5 Gm. of sodium citrate per day) for a period of at least seven days before the experiments were undertaken. It was customary to withhold food for eighteen to twenty-four hours before collecting the samples for analysis. Control determinations of the plasma volume and the "available (thiocyanate) fluid" were made by the direct method of Gregersen and Stewart,[14] as adapted to the photoelectric colorimeter by Gibson and Evelyn.[15] The animals then received desoxycorticosterone acetate* subcutaneously (1 mg. per kilogram of body weight daily) for a period of fourteen days, at which time the above determinations were repeated. Immediately following these determinations, blood was withdrawn under oil from the femoral artery, placed under oil in a special centrifuge tube containing heparin to prevent clotting, and centrifuged at once for the plasma analyses. Skeletal muscle (lumbar portion, sacrospinalis) was obtained under sodium pentobarbital anesthesia as quickly as possible following the collection of the blood and was sampled for the several analyses in the manner previously described.[16] The following determinations were carried out on the plasma: water, chloride, sodium and potassium; on the muscle: water, chloride, sodium, potassium, and total neutral fat. The chemical methods were the same as those employed in previous studies.[17]

The administration of the desoxycorticosterone acetate was then discontinued, and after a period of fourteen days the foregoing observations were repeated. During this interval the diet remained unchanged in three of the animals while in five others the salt supplement was changed to 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate daily.

The volumes of the extracellular (F) and intracellular (C) phases of muscle were calculated in the manner outlined by Hastings and Eichelberger,[18] which is based upon the assumption that all the chloride is extracellular and is present at a concentration equal to that of an ultrafiltrate of plasma. In order to approximate the volume changes produced in the extracellular and intracellular phases of 1 kilogram of original muscle, the calculations were made relative to a control series, assuming a constant solid content of the intracellular phase. The partition of sodium and potassium between the extracellular and intracellular phases of muscle was carried out by the method described by previous authors.[18, 19] The experimental values herein reported for intracellular sodium and potassium are given in terms of a kilogram of intracellular water and have been corrected to an intracellular solid content equal to that of the control mean.

## RESULTS

*Changes of the Plasma Volume and "Available (Thiocyanate) Fluid."*— Measurements of plasma volume and "available (thiocyanate) fluid" were carried out on nine animals following the administration of desoxycorticosterone acetate and on eight animals after its discontinuance. On the basis of individual control levels, the plasma volume was found to be increased by an average of 11 per cent and to range from 1 to 33 per cent, with an increase greater than 10 per cent occurring in four instances. (The increase of plasma volume amounted, on the average, to 77 c.c., with a range of variation from 8 to 206 c.c.). Likewise the "available (thiocyanate) fluid" was found to be increased

*We are indebted to the Schering Corporation for the generous supply of crystalline desoxycorticosterone acetate in sesame oil (Cortate) which was employed for these studies.

by an average of 15 per cent and to range from 5 to 40 per cent, with an increase greater than 10 per cent occurring in five instances. (The increase of "available (thiocyanate) fluid" amounted, on the average, to 700 c.c., with a range of variation from 290 to 1,600 c.c.)

Fourteen days after the administration of the drug had been discontinued, there was found to be a persistence of the average increase of the plasma volume and, although less, of the "available (thiocyanate) fluid." Thus the plasma volume was found to be increased by an average of 16 per cent and to range from 2 to 42 per cent, with an increase greater than 10 per cent occurring in five instances. Similarly, the "available (thiocyanate) fluid" was found to be increased, on the average, by 7 per cent and to range from –5 to 21 per cent, with values greater than 10 per cent occurring in only three instances.

From these data it would appear that, although in a fairly high percentage of animals receiving large doses of desoxycorticosterone acetate the increases of plasma volume and "available (thiocyanate) fluid" may not exceed the possible experimental error, there does seem to be a definite tendency for both volumes to become elevated and in some instances to become elevated appreciably.

TABLE I

ELECTROLYTE AND WATER CONTENT OF PLASMA FROM DOGS FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE AND AFTER ITS DISCONTINUANCE*

| | WATER | CHLORIDE | SODIUM | POTASSIUM |
|---|---|---|---|---|
| | Gm. per kg. | meq. per kg. water | | |
| Controls (17) | | | | |
| Mean | 918.4 | 120.5 | 156.3 | 3.1 |
| Standard deviation | 7.1 | 2.9 | 3.5 | 0.6 |
| Following desoxycorticosterone acetate (10) | | | | |
| Mean | 920.2 | 114.6 | 157.4 | 2.1 |
| Maximum | 926 | 118 | 161 | 3.1 |
| Minimum | 915 | 111 | 152 | 1.6 |
| After desoxycorticosterone acetate discontinuance (3)† | | | | |
| Mean | 914.5 | 117.1 | 159.2 | 2.2 |
| Maximum | 920 | 118 | 163 | 2.7 |
| Minimum | 908 | 116 | 156 | 2.0 |
| After desoxycorticosterone acetate discontinuance (5)‡ | | | | |
| Mean | 918.3 | 116.4 | 156.3 | 2.6 |
| Maximum | 923 | 119 | 162 | 3.4 |
| Minimum | 913 | 115 | 153 | 2.2 |

*Two-week periods. One milligram per kilogram of body weight of desoxycorticosterone acetate was administered daily. The numbers within the parentheses indicate the number of animals.

†High sodium and low potassium diet.

‡Low sodium and moderately high potassium diet.

*Plasma Changes.*—The results of the analysis of plasma are presented in Table I. By comparison with a control series[2] it will be seen that the outstanding changes encountered following the administration of desoxycorticosterone acetate were the deficits in the average levels of potassium and chloride. That there was a tendency to an elevated plasma sodium concentration was indicated by the slightly elevated average value.

Two weeks after the administration of the drug had been discontinued, the plasma sodium showed, on the average, a further increase in the three animals whose diet was left unchanged, while it fell back to the control mean in the

five animals whose salt supplement was changed to 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate daily. Some decrease of the average levels for both potassium and chloride was still found to be present in both groups of animals.

Attention should be called to the fact that Kuhlman, Ragan, and associates,[12] and Ferrebee, Parker, and others[10] noted an increase of the plasma sodium concentration amounting to about 4 or 5 meq. per liter and a fall of the plasma potassium of about 1 to 1.5 meq. per liter in normal dogs receiving 25 mg. of desoxycorticosterone acetate daily for periods of twenty or more days. Although the changes of the plasma sodium were less striking, the deficits of plasma potassium encountered in the present experiments are of the order noted by the above workers. It is of some interest that even though the plasma potassium tended to return to normal, the average figure was still lowered somewhat two weeks after the discontinuance of the drug despite the added daily intake of potassium.

TABLE II

ELECTROLYTE AND WATER CONTENT OF SKELETAL MUSCLE FROM DOGS FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE*

(The values are expressed in terms of 1,000 Gm. of fat-free muscle.)

| | CONTROLS (17) | | FOLLOWING DESOXYCORTICOSTERONE ACETATE ADMINISTRATION (10) | | |
|---|---|---|---|---|---|
| | MEAN | STANDARD DEVIATION | MEAN | MAXIMUM | MINIMUM |
| Chloride, meq. | 19.8 | 2.0 | 20.0 | 22 | 19 |
| Sodium, meq. | 31.1 | 3.1 | 46.1 | 57 | 39 |
| Potassium, meq. | 88.5 | 6.0 | 66.5 | 76 | 52 |
| Water, Gm. | 764.0 | 8.4 | 759.8 | 776 | 749 |
| Extracellular phase, (F), Gm. | 158 | 15 | 168 | 180 | 146 |
| Intracellular water, $[H_2O]_c$,† Gm. | 722.3 | 9.7 | 713.8 | 732 | 702 |
| Intracellular sodium, $[Na]_c$,‡ meq. | 12.7 | 4.7 | 35.6 | 54 | 21 |
| Intracellular potassium, $[K]_c$,‡ meq. | 145.0 | 11.7 | 111.2 | 124 | 88 |
| ΔM, Gm. | | | -16 | -60 | 55 |
| ΔC, Gm. | | | -23 | -59 | 30 |
| ΔF, Gm. | | | 7 | 30 | -19 |

*Lumbar portion, sacrospinalis. One milligram per kilogram of body weight of desoxycorticosterone acetate was injected daily for fourteen days.

†Per 1,000 Gm. of muscle cells.

‡Per 1,000 Gm. of intracellular water, corrected to a solid content equal to that of the control. Numbers within the parentheses indicate the number of animals.

*Skeletal Muscle Changes.*—The results of the analysis of skeletal muscle have been presented in Tables II and III. By comparing these data with those of a control series,* it will be observed that two weeks following the administration of desoxycorticosterone acetate (Table II) there was a striking fall of skeletal muscle potassium and a gain of sodium. The muscle chloride remained unchanged while the average water content was lowered slightly.

The partition of sodium and potassium between the extracellular and intracellular compartments of skeletal muscle revealed that the "excess" sodium, which can be located in the intracellular phase, $[Na]_c$, increased from a control level of 12.7 meq., ±4.7 meq., per kilogram of intracellular water to an average figure of 35.6 meq., with a range of variation from 21 to 54 meq. Simultaneously, the average intracellular potassium per kilogram intracellular water, $[K]_c$

*The data for plasma and skeletal muscle of the control series were reported in connection with another study.[17] These observations were carried out on a separate series of animals; however, the chemical methods and management of the animals were identical with those employed here.

fell from the control level of 145.0 meq., ±11.7 meq., to 111.2 meq., with a range of variation from 88 to 124 meq. It will be observed that, on the average, the sum of the concentrations $[Na]_c$ and $[K]_c$ become lowered by 10.9 meq., indicating that the gain of "excess" sodium did not completely compensate the loss of intracellular potassium. In this connection it will be seen that the calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilogram of control muscle revealed some irregularity. However, on the basis of an average 23 Gm. decrease in the intracellular phase, $\Delta C$, and an average amounted to a decrease of 16 Gm. per kilogram of control muscle, $\Delta M$, consisting of an average 23 Gm. decrease in the intracellular phase, $\Delta C$, and an average 7 Gm. increase in the extracellular phase, $\Delta F$. Thus, despite a marked divergence from normal of the electrolyte pattern of the intracellular phase, there were, on the average, no striking changes from normal in the volume relationship between the extracellular and intracellular phases. In fact, the average volume changes encountered were in the direction of those which might be anticipated on the basis of a gain of the extracellular sodium concentration and of the "available (thiocyanate) fluid."

TABLE III

ELECTROLYTE AND WATER CONTENT OF SKELETAL MUSCLE FROM DOGS AFTER THE DISCONTINUANCE OF DESOXYCORTICOSTERONE ACETATE ADMINISTRATION*

(The values are expressed in terms of 1,000 Gm. of fat-free muscle.)

| | HIGH SODIUM—LOW POTASSIUM DIET (3) | | | LOW SODIUM—MODERATE POTASSIUM DIET (5) | | |
|---|---|---|---|---|---|---|
| | MEAN | MAXIMUM | MINIMUM | MEAN | MAXIMUM | MINIMUM |
| Chloride, meq. | 20.7 | 23 | 18 | 20.0 | 24 | 16 |
| Sodium, meq. | 46.9 | 53 | 39 | 40.1 | 45 | 30 |
| Potassium, meq. | 61.4 | 63 | 60 | 72.5 | 83 | 64 |
| Water, Gm. | 762.0 | 775 | 748 | 765.5 | 773 | 759 |
| Extracellular phase, (F), Gm. | 170 | 183 | 147 | 165 | 193 | 135 |
| Intracellular water, $[H_2O]_c$,† Gm. | 715.8 | 728 | 707 | 721.2 | 732 | 711 |
| Intracellular sodium, $[Na]_c$,‡ meq. | 35.9 | 42 | 29 | 25.9 | 36 | 15 |
| Intracellular potassium, $[K]_c$,‡ meq. | 102.7 | 104 | 99 | 119.6 | 132 | 105 |
| $\Delta M$, Gm. | -6 | -66 | 49 | 7 | 41 | -22 |
| $\Delta C$, Gm. | -17 | -45 | 19 | -1 | -35 | 34 |
| $\Delta F$, Gm. | 11 | 30 | -21 | 8 | 37 | -21 |

*Lumbar portion, sacrospinalis. Observations were made fourteen days after discontinuance of desoxycorticosterone acetate administration.

†Per 1,000 Gm. of muscle cells.

‡Per 1,000 Gm. of intracellular water, corrected to a solid content equal to that of the control. Numbers within the parentheses indicate the number of animals.

By comparing the data presented in Table III with those given in Table II, it will be observed that a marked deviation from normal of the skeletal muscle sodium and potassium content was found to persist in the animals studied two weeks after the administration of the drug had been discontinued. The partition of sodium and potassium between the extracellular and intracellular compartments revealed, on the average, no tendency of the intracellular concentrations of these two cations to return to normal levels in the three animals which were permitted to continue on the diet high in sodium and low in potassium. However, in the case of the five animals which received a daily salt supplement of 3 Gm. of sodium chloride plus 10 Gm. of potassium dihydrogen phosphate during the period of drug discontinuance, a tendency for the intracellular concentrations of sodium and potassium to return to normal was noted. Thus in the latter animals

the average intracellular sodium, $[Na]_c$, was 9.7 meq. lower, and the average intracellular potassium, $[K]_c$, was 8.4 meq. higher than the average levels observed after the administration of the drug. It will be observed that as was the case with the muscles of the animals examined after the administration of the desoxycorticosterone acetate, the calculations of the volume changes produced in the extracellular and intracellular phases of 1 kilogram of control muscle revealed, on the average, no changes from normal which could not be anticipated on the basis of the extracellular sodium concentration and the "available (thiocyanate) fluid."

## DISCUSSION

At the time the present experiments were being brought to a conclusion, the report by Ferrebee, Parker, Carnes, Gerity, Atchley, and Loeb[10] appeared, calling attention to the fact that normal dogs receiving daily injections of 25 mg. of desoxycorticosterone acetate develop muscular weakness and a syndrome resembling diabetes insipidus. It was pointed out, further, that there were attacks of paralysis associated with a partial replacement of the intracellular potassium of the skeletal muscles by sodium. In respect to the distortion from normal, the changes of intracellular sodium and potassium of skeletal muscle encountered in the present study are in complete harmony with those reported by the aforementioned workers. Even though smaller doses were employed in the present experiments, two of the ten dogs exhibited paralysis while receiving the drug. Interestingly enough, these two animals also showed the greatest losses of intracellular potassium and gains of intracellular sodium, the figures for $[K]_c$ being 87.7 and 96.0 meq., and those for $[Na]_c$ being 54.4 and 47.4 meq. per kilogram of intracellular water, respectively.

## SUMMARY

Changes of the plasma volume, the "available (thiocyanate) fluid," and the electrolyte and water content of plasma and skeletal muscle were observed in normal dogs following the daily injection (1 mg. per kilogram of body weight) of desoxycorticosterone acetate for a period of two weeks and two weeks after the administration of the drug had been discontinued. The pertinent findings may be summarized as follows:

1. Normal dogs maintained on a high sodium-low potassium diet exhibited a tendency for both the plasma volume and the "available (thiocyanate) fluid" to become increased while receiving the drug for a period of two weeks. In many instances the increases of plasma volume and "available (thiocyanate) fluid" did not exceed the possible experimental error. However, not infrequently both volumes were found to be elevated appreciably.

2. Two weeks after the administration of the drug the animals showed, on the average, a fall of the plasma potassium and chloride concentrations and a tendency toward an elevated plasma sodium concentration. At the same time there was a striking reduction of the skeletal muscle potassium content, a gain of sodium, and no change of the skeletal muscle chloride. On the average, the loss of intracellular potassium was not completely compensated by a gain of the sodium which could be located in the intracellular phase.

3. The animals which were studied two weeks after the administration of the drug had been discontinued continued to show a marked reduction of the skeletal

muscle potassium and elevation of sodium. While there was no tendency for the intracellular concentrations of these two cations to return to normal levels in animals maintained on a high sodium-low potassium diet during this period, there was, on the average, such a tendency in the animals receiving a lowered sodium and increased potassium intake.

4. Despite the marked divergence of the electrolyte pattern of the intracellular phase from the normal, there were, on the average, no striking changes from normal in the volume relationship between the extracellular and intracellular phases.

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# LABORATORY METHODS

## GENERAL

### USE OF SODIUM DESOXYCHOLATE-LYSED ANTIGEN FOR THE PRODUCTION OF PNEUMOCOCCUS ANTISERUM*

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THE production of antibodies against pneumococci by the use of lysates of pneumococci has been demonstrated by a number of investigators. Meyer,[1] and Meyer and Sukneff,[2] used a sodium taurocholate lysate of type I pneumococci to immunize rabbits and mice. Pneumococci treated with soaps (sodium ricinoleate) were used by Larson and Nelson[3] and by Larson[4] for prophylaxis in man and to produce a therapeutic antiserum. Pneumococci treated with bile salts were used as antigens by some workers.[5] White,[6] summarizing previous work, says: "Filtrates from fluid cultures and watery or saline extracts of pneumococci, representing as they do only a part of the antigenic components of the pneumococcal cell, are more limited in immunizing properties than the entire cell," and further, "while pneumococci treated with bile salts may induce immunity in mice and rabbits, and presumably in other animals, the immunity is of low order and is lacking in type specificity."

Our experiments were made with antigens prepared in three ways: 1. *Heat-killed whole cultures* of young virulent strains of type I pneumococci were made into antigens according to the method of the Massachusetts Antitoxin and Vaccine Laboratory (quoted by White). 2. *Lysate antigens,* made from sixteen-hour broth cultures of the same strains, washed with salt solution, and diluted with salt solution until the turbidity of the suspension corresponded to tube 3 of a barium sulfate standard.[8] The suspension was then lysed by adding 2 drops of sodium desoxycholate solution[9] to each 8.0 c.c. of the culture suspension. 3. *Lysate antigens,* prepared as described and *passed through a Seitz filter* to remove any possibility of production of antibodies being the result of particles of the cells that failed to dissolve. All antigens were prepared fresh daily.

Some of the rabbits were given intravenous injections of antigens for twelve consecutive days; an initial injection of 0.5 c.c., increased each day by 0.5 c.c., until a maximum daily dose of 2.0 c.c. was attained. One group of rabbits was injected according to the protocol of the Massachusetts Antitoxin and Vaccine Laboratory, consisting of a total of 18 injections given over a period of twelve weeks. Other rabbits were given a total of 12 injections in series of four con-

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This work was supported in part by a grant from Hynson, Westcott & Dunning, Baltimore.

Received for publication, May 5, 1942.

secutive days with seven-day rest periods between series. In the above experiments no filtered-lysed antigen was used. In still another group of rabbits, however, two animals were injected with heat-killed antigen, two with lysed antigen, and two with filtered-lysed antigen. Four daily injections were given, followed by a rest period of thirty-one days and four more daily injections. All animals were bled five days after the last injection. No significant difference was obtained when the same type of antigen was used in the above methods of immunization. It would appear, therefore, that the shorter period of immunization is to be preferred.

## RESULTS

The potency of the antisera was determined by means of agglutination tests, Neufeld tests, microscopic precipitin tests by the method of Schaub and Reid,[7] and mouse protection tests according to the potency (of serum) test of the National Institute of Health. The results can be summarized in Table I. They were similar in all trials except for individual variations in the rabbits.

TABLE I

| TEST | HEAT-KILLED ANTIGEN | LYSED PNEUMOCOCCUS ANTIGEN | LYSED AND FILTERED ANTIGEN |
|---|---|---|---|
| Agglutination tests | Complete in 1:320 dilution | Complete in 1:320 dilution | Not done |
| Neufeld tests | Slight "Quellung" | Slight "Quellung" | Slight "Quellung" |
| Microscopic precipitin tests | Unsatisfactory | Unsatisfactory | Unsatisfactory |
| Mouse protection tests | Protection against as much as 10 million L.D. | Protection against as much as 1 million L.D. | Protection against as much as 1 million L.D. |

## DISCUSSION

The agglutination and mouse protection titers of sera from rabbits injected twelve times on consecutive days with lysate antigen compared favorably with that of sera from rabbits given a heat-killed antigen either as 18 injections over twelve weeks or 12 injections on as many days. Four lysate immunized animals gave lower titers than any of those immunized with heat-killed antigen. In a couple of instances lower titers were obtained in animals immunized with heat-killed antigen than were obtained in those giving best results with lysed antigen. Animals immunized with filtrate from lysed cultures produced as high titer serum as those injected with lysate not filtered.

The specificity of antiserum produced in rabbits by injecting sodium desoxycholate-lysed antigen is nearly equal, in these experiments, to that of antiserum produced in rabbits with heat-killed antigens.

No apparent systemic harm was done to the rabbits by the sodium desoxycholate. Local necrosis and edema occurred when the sodium desoxycholate antigens entered directly into the tissues of the rabbit.

## CONCLUSIONS

1. Sera from rabbits immunized with lysate, filtered or not, freshly prepared by treating young virulent cultures of type I pneumococci with sodium desoxy-

cholate, gave specific mouse protection and showed some ability to cause agglutination and "Quellung" in specific type sera but gave no group reaction.

2. The sodium desoxycholate-lysed pneumococci produced, in a short time, in rabbits, antisera of high titer and possessing type specificity.

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# THE CYTOLOGY OF THE POLYMORPHONUCLEAR LEUCOCYTE IN TOXIC CONDITIONS*

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FROM time to time it has been suggested that valuable information can be obtained by considering the detailed cytology of the white blood cells in infective states, altogether apart from that given by the form of the polymorph nuclei, which is the basis of the Arneth count, the polynuclear count, and their modifications. More specifically, many observers from the time of Bodkin (1892) and Ewing (1901) have been impressed by the degenerative changes in the polymorphs seen in severe toxemias, and Strumia (1936) has classified the degenerated cells into those showing "toxic granules" (Cesaris-Demil, 1908), those with clumped granules and vacuolation, and those showing "coagulation necrosis," edema, and nuclear rupture. Taking these cytological appearances along with the "nonfilamented count" (a simplified polynuclear count, the nonfilamented cells being equivalent to cells of Class I) Amidon (1939) has tried to relate the changes in white blood cell cytology to the clinical course in toxic states (appendicitis, pneumonia, etc.), but is not enthusiastic about the practicability of the procedure, nor certain of the significance of the results.

No one who examines large numbers of properly stained blood films for the purpose of making routine differential or polynuclear counts can fail to be impressed by the variations in white blood cell cytology which present themselves,

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Received for publication, May 19, 1942.

but the difficulty is to express what one sees in such a way as to give the various cytological appearances (toxic granulation, vacuolation, etc.) their proper weight, so that the final result is in some way related to the clinical state of the patient. As an example of the difficulty, one soon finds that it is quite unsatisfactory to give the percentage number of cells with toxic granulations as opposed to the number without them, for the division is not sufficiently distinct, and the cells with toxic granules vary greatly with respect to the degree of granulation. Similarly, an enumeration of degenerated, as opposed to undegenerated, cells does not allow for the fact that there are many degrees of degeneration. Still further difficulty results when one considers two attributes, such as toxic granulation and degeneracy, together, for a degenerate cell may or may not have toxic granules; such attempts at an all-or-none classification only lead to Amidon's conclusion that attention to these cytological details is more trouble than it is worth.

These difficulties largely disappear if one gives up trying to enumerate all-or-none characteristics (percentage of vacuolated cells, percentage of cells with toxic granules, etc.), and substitutes an estimate of the extent to which each abnormal attribute (vacuolation, toxic granulation, etc.) appears in the average cell, describing the extent by plus signs or numbers, and forming an impression of the "average cell" while examining the film for other purposes, e.g., while making a differential count or polynuclear count.* This report is concerned with the results of applying this method to several thousand blood films over about a year's time, during which the method was being tested for its practicability.

## STAINING METHODS

1. *Preparation of glassware.* New, or at least unetched, slides are washed in a strong solution of Gold Dust for hours or even days, and are rinsed thoroughly and individually in hot running water. They are then dipped in 95 per cent alcohol, wiped dry with a clean towel, and stored in covered boxes. Cover slips are cleaned in the same way. One should not use cleaning solution or acid alcohol, because anomalies in the staining reaction follow.

2. *Staining.* Films are made in the usual way from fresh blood, and with properly cleaned slides it is easy to prepare thin and even films. Heparinized blood can be used if fresh, but oxalated blood should not be used. The film is allowed to dry in air, and is fixed for five minutes in methyl alcohol in a Coplin jar. The methyl alcohol must be "absolute, acetone free," and should be frequently replaced; if this is not done, anomalies in staining result. The fixed film is dried again in air before the application of the stain. Giemsa blood stain, stock solution, is diluted 1:10 with distilled water, the dilution being made just

*Osgood and Ashworth illustrate toxic neutrophiles in their *Atlas of Hematology*, Nos. 87 to 92. They describe three cytological changes in toxic conditions: basophilia of cytoplasm, the presence of vacuoles, and the presence of toxic granules. These they grade from one plus to four plus, and they say that death occurred in 100 consecutive cases in which two of these factors were graded from three to four plus. On the system of grading described in this paper, three plus and four plus are of less serious prognostic significance than on theirs. It will be noticed that their system does not include a grading of the feature "pyknotic nuclei," because this feature is not so clearly observed with Wright's stain, which Osgood and Ashworth use. They continue: "Evaluation of toxic changes is more reliable in prognosis than either the staff cell count or the ratio of neutrophiles to total polymorphs, but the use of all these criteria is worth while. Rosenthal and Sutra count the percentage of polymorphs showing toxic granules and report the result as the 'degenerative index.' We have had better results by evaluating all three toxic changes, and it is simpler to report these on a scale of one plus to four plus as one is doing the differential count." This corresponds to our experience.

before the stain is to be used; buffering is unnecessary if the water is freshly distilled. The slide, placed film-side up on a staining rack, is flooded with the diluted stain. The duration of staining is most important, and should be neither more nor less than thirty minutes, at the end of which time the film is rinsed with running distilled water for fifteen seconds, flooded with distilled water for thirty seconds, and finally tipped up on end to dry in air. The back of the slide is polished while it is still wet, and the examination of the cells is carried out with an oil-immersion objective in the usual way on the unmounted film. The slightest variation from this standard procedure will result in changes in the staining reaction, and, as a control on the staining method, one may use a film of one's own blood.

### GRADING OF CYTOLOGICAL FEATURES

One hundred polymorphs are examined, as in the making of a polynuclear count, which, indeed, is done on every film, as described by Cooke and Ponder (1928). As each polymorph is seen, it is carefully examined and mentally classified as regards four features other than nuclear configuration: (1) whether the cytoplasmic granules are large, separated, and deeply staining, metachromatic or azurophile ("toxic granulation"); (2) whether the cell outline is sharp and circular, or irregular and presenting pseudopods ("ameboid"); (3) whether the cytoplasm of the cell is vacuolated ("vacuolation"); and (4) whether the nucleus shows pyknotic areas ("pyknotic nuclei"). After such a survey the observer will be able to form an opinion of the cytology of the average cell with respect to four features; toxic granulation, ameboid, vacuolation, and pyknotic nuclei. He will be able to give a somewhat arbitrary grade to each of these features, from one plus to four plus, and it is a matter of experience that he can do so with a surprising degree of certainty.

All sorts of elaborate schemes could be devised for expressing the results of the survey, but we have found it sufficient to denote the occurrence of each abnormal feature by a plus sign (from one plus to four plus), and to add the number of plus signs together, averaging the number of plus signs for the features "ameboid" and "vacuolated." The result of the survey is then represented by a figure which varies from 0 to 12, the value 0 meaning that all the abnormal cytological features are absent, and the value 12 meaning that they are each present to a maximal extent. For example, if a film shows:

| | |
|---|---|
| Toxic granulation | +++ |
| Ameboid | + |
| Vacuolation | + |
| Pyknotic nuclei | ++ |

it is described as a film of grade 6. Again, if a film shows the following features:

| | |
|---|---|
| Toxic granulations | ++++ |
| Ameboid | ++ |
| Vacuolation | ++++ |
| Pyknotic nuclei | ++ |

it is described as a film of grade 9. A normal film is obviously one of grade 0. This method of grading the films may seem rough and arbitrary, but, nevertheless, it expresses the results of the cytological survey in a remarkably satisfactory way.

## SIGNIFICANCE OF THE CYTOLOGICAL CHANGES

Considerable experience in grading polymorphs has brought out a number of points which have a bearing on the significance of the cytological changes.

1. The changes are not of an all-or-none nature, some cells showing them and some not. They affect all the cells, although some more than others, and this results in the information given by the grade being very different from that given by the polynuclear count. The figures for the various classes of the polynuclear count give the proportion of young cells (Class I) to older cells (Classes II, III, IV, and V, in order of increasing age), and a left-handed deflection shows that there has been either an increased production of young cells as a result of marrow stimulation, or an increased destruction of the older cells, or a combination of the two. This corresponds, by analogy with what occurs in a population, to an increased birth rate, an increased death rate in the older classes, or to a combination of both, and is a change which is characteristic of the population as a whole rather than of any one member in it. The grade, on the other hand, is an expression of the average state of the cell, not as regards age, but as regards cytological appearance and the processes which determine it at the moment; to extend the analogy, this would roughly correspond to the average nutrition of the individuals of the population. Whereas a change in the polynuclear count expresses a disturbance of the production-destruction steady state, a change in the grade seems to be evidence of an alteration in the *milieu* in which the cells circulate, and this produces substantially the same changes in all the cells simultaneously. An excellent example of this can be seen in cases where films are made within a few hours of an accident resulting in extensive tissue damage; e.g., compound fracture of femora, two hours after accident: 29,700 leucocytes per cubic millimeter, 81 per cent polymorphs, polynuclear mean, 1.41, grade 6.* The cells of all the polynuclear classes, from I to V, and even polycytes if they are present, show the changes in cytology, although the large number of cells of Class I are young cells just liberated from the marrow, while the cells of the higher classes must have been normal inhabitants of the blood stream at the time of the accident. Perhaps the best of all instances of the rapid development of pyknotic nuclei in the polymorphs of all the polynuclear classes is to be found in the guinea pig after the injection of nucleic acid (Curphey and Ponder, 1941); the multilobulated polymorphs (as high as Class X) all develop pyknotic nuclei within an hour of the subcutaneous injection of 1 mg. of nucleic acid per kilogram, and regain their normal appearance from four to eight hours later.

2. Deflections of the polynuclear count without anything more than the most trifling change in the grade (e.g., from 0 to 1, or from 1 to 2) occur after inhalation anesthesia (ether, ethylene, etc.) and can be discounted.

3. While a left-handed deflection of the polynuclear count is an excellent measure of the severity of an acute infection, and while a subsequent right-handed shift is a reliable indication of recovery, it is well recognized that the count tends to become stabilized at a low figure (usually 1.2 to 1.5) in chronic infections or in toxic states of long duration. Under these circumstances the

*This case may be contrasted with one of hemorrhage from the radial artery, uncomplicated by toxic manifestations: 21,000 leucocytes per cubic millimeter, 90 per cent polymorphs, polynuclear mean 1.28, grade 0.

fixed polynuclear count does not reflect small changes in the severity of the toxemia, and the grade is a far more sensitive measure of variations in the toxic state. Changes in the grade from day to day are of great prognostic value, especially if there is a definite trend, and although these changes are ultimately followed by corresponding shifts in the polynuclear count, they constitute the better guide to progress and indication of the outcome (cf. footnote,* page 317).

4. The various cytological features do not always undergo alteration simultaneously. The appearance of vacuoles, however, is so constantly accompanied by an ameboid cell outline that the abnormal properties "vacuolation" and "ameboid" can be considered as two manifestations of the same underlying change, and each given only half the weight of an abnormal attribute in determining the grade. "Pyknotic nuclei" often occur without any other abnormal cytological feature being present, and almost invariably do so when the patient becomes either dehydrated or acidotic.

5. There are certain artifacts which may lead to misinterpretations if unrecognized. The most important of these are the artifacts introduced by variations in the staining method, and it cannot be too strongly emphasized that the system of grading described here is entirely dependent on the staining methods being carried out without modification. The most frequently occurring artifacts are the following: (a) On improperly cleaned slides, and particularly on slides cleaned with acid, cleaning solution, and acid alcohol, the cytoplasm appears pink and the nuclei show pyknotic areas. (b) Methyl alcohol which is not fresh produces the same artifacts as are seen on slides cleaned with acid solutions. (c) Films made from blood kept for some hours in the refrigerator are likely to show cells which are vacuolated and ameboid. (d) Films made from oxalated blood show shrunken white blood cells, the cytology of which is very different from that of cells from fresh or heparinized blood. (e) Cells in films from patients who have been treated with sulfonamide drugs, and particularly sulfanilamide, are ameboid and vacuolated.

## CLINICAL ILLUSTRATIONS

Tables I, II, and III illustrate the results of using this method of grading.

Table I shows the total white blood cell count, the polymorphs percentage, the polynuclear count mean, and the grade in patients who showed no signs of toxemia clinically. I have used the preoperative figures for 12 consecutive cases in which partial gastrectomy or colectomy was performed, since these provide a good illustration of what is found in surgical cases without toxic manifestations. Inspection of Table I shows that the grade, rather than the total white cell count, the polymorph percentage, or the mean of the polynuclear count, is the reliable indication of the absence of toxemia.

Table II gives the same data in 12 cases in which there were clinical signs of severe toxemia. Again it will be apparent that the grade is a better indication of the toxic condition than the total white blood cell count, the polymorph percentage, or the polynuclear count.

Table III shows the values for the grade in a series of miscellaneous toxic conditions (corresponding roughly to Amidon's Table IV). By "first observation" is meant the observation on admission or at the time of the acute episode.

TABLE I

| | TOTAL COUNT × $10^{-3}$ | POLYMORPHS % | POLYNUCLEAR MEAN* | GRADE |
|---|---|---|---|---|
| Carcinoma of cecum | 11.2 | 69 | 2.21 | 0 |
| Carcinoma of rectum | 9.3 | 54 | 2.20 | 0 |
| Stenosing gastric ulcer | 4.7 | 42 | 2.16 | 1 |
| Stenosing gastric ulcer | 7.4 | 79 | 2.00 | 0 |
| Stenosing gastric ulcer | 9.2 | 58 | 1.98 | 0 |
| Carcinoma of sigmoid | 6.1 | 74 | 1.78 | 0 |
| Stenosing gastric ulcer | 8.4 | 64 | 1.75 | 1 |
| Stenosing gastric ulcer | 10.2 | 60 | 1.66 | 1 |
| Carcinoma of sigmoid | 7.7 | 65 | 1.66 | 1 |
| Endometriosis of cecum | 14.3 | 76 | 1.64 | 2 |
| Carcinoma of hepatic flexure | 5.9 | 64 | 1.39 | 3 |
| Carcinoma of pylorus | 14.1 | 82 | 1.36 | 2 |

*By the polynuclear mean is meant the average number of lobes per polymorph. It is computed in a count of 100 polymorphs by multiplying the number of cells with one lobe to the nucleus by 1, the number with two lobes by 2, the number with three lobes by 3, the number with four lobes by 4, and the number with five or more lobes by 5; the figures are added together and divided by 100. The resulting figure expresses in a general sort of way, what is expressed by writing out the figures for the count in extenso. The normal polynuclear mean ranges from 2.3 to 2.7; means less than this correspond to "left-handed deflections," and the most extreme deflection (100 cells of Class I) corresponds to a mean of 1.0

TABLE II

| | TOTAL COUNT × $10^{-3}$ | POLYMORPHS % | POLYNUCLEAR MEAN | GRADE |
|---|---|---|---|---|
| Toxemia of pregnancy | 7.6 | 66 | 2.49 | 10 |
| Pelvic inflammatory disease | 8.5 | 60 | 2.24 | 7 |
| *Subacute endocarditis | 3.9 | 81 | 1.77 | 9 |
| *Subphrenic abscess | 20.2 | 86 | 1.74 | 8 |
| Diffuse peritonitis | 17.4 | 93 | 1.71 | 9 |
| Tuboovarian abscess | 29.4 | 87 | 1.66 | 8 |
| Acute pancreatitis | 30.6 | 83 | 1.46 | 9 |
| Rectovesical fistula | 10.4 | 78 | 1.44 | 7 |
| *Diverticulitis | 20.1 | 68 | 1.34 | 11 |
| *Appendicitis, peritonitis | 27.5 | 59 | 1.31 | 10 |
| *Gangrene of ileum | 5.9 | 79 | 1.20 | 10 |
| *Retroperitoneal hemorrhage; ruptured bladder; pneumonia | 8.2 | 89 | 1.16 | 10 |

*Died.

TABLE III

| | OBSERVATION | | INTERVAL IN DAYS |
|---|---|---|---|
| | FIRST | FINAL | |
| Frostbite, both feet. Demarcation with loss of toes under conservative treatment. Uneventful recovery | 3 | 1 | 21 |
| Bullet wound of chest; hemothorax. Uneventful recovery | 1 | 0 | 8 |
| Perinephritic abscess. Drainage. Uneventful recovery | 4 | 1 | 7 |
| Volvulus of ileum. Resection with ileostomy. Slow convalescence | 6 | 2 | 19 |
| Incomplete abortion. Pelvic thrombophlebitis following curettage. Recovered | 5 | 1 | 12 |
| Ruptured appendix with peritonitis. Uneventful recovery | 5 | 0 | 10 |
| Nephrolithiasis. Operation followed by pneumothorax and collapse of right lung; pneumonitis. Recovered | 8 | 2 | 20 |
| Gangrenous appendix with peritonitis. Died on fourth day | 5 | 10 | 4 |
| Intestinal obstruction due to adhesions. Operation followed by aspiration pneumonia. Died | 6 | 9 | 3 |
| Multiple fractures; retroperitoneal hemorrhage; anuria. Died | 4 | 12 | 4 |

and by "final observation" is meant the observation at the time of discharge or death. The interval in days is the interval between the two observations, but it ought to be pointed out that in actual practice observations are made daily, and sometimes more frequently. Table III illustrates the fact that an improvement in the grade corresponds to a good prognosis, while the abnormal cytological features become more prominent as the clinical condition becomes worse. This conclusion is borne out by an experience which now extends to an examination of several thousand blood films.*

## CONCLUSIONS

1. In toxic conditions the polymorphs show a number of cytological changes, the principal ones observed with Giemsa stain being toxic granules, an ameboid outline, cytoplasmic vacuoles, and pyknotic areas in the nucleus. A simple method is described for grading the cells of a film according to the degree to which each of these abnormal appearances is developed, the grade varying from 0 for normal cells to 12 for the polymorphs seen in extremely toxic conditions.

2. The grade assigned by this method closely reflects the clinical condition of the patient, much more so than the total white blood cell count, the polymorph percentage, the "degenerative index" (which it replaces), or even the polynuclear count. Changes in the grade taken together with changes in the polynuclear count are of real prognostic value.

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*Amidon (1939) remarks that the information gained from various hematological methods of examination must be balanced against the time, effort, and equipment required, and concludes that the determination of the degenerative index, in particular, is impracticable because it requires a degree of training and technical skill not to be expected of the average hospital technician. In our experience there is no greater difficulty in grading white blood cells than there is in doing a differential white blood cell count on abnormal blood or in doing a polynuclear count. Even the beginner is not likely to have much difficulty if he once realizes that it is essential to standardize the staining method.

# IS THE MAINTAINED SEDIMENTATION RATE SPECIFIC FOR MALIGNANCY?*

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WITH THE TECHNICAL ASSISTANCE OF HELEN BERNSTEIN

IN 1937 Koster[1] drew attention to the fact that the sedimentation rate of erythrocytes in stored citrated blood is subject to changes; that is, that over a twenty-four-hour period the blood sedimentation rate shows a tendency to decrease. However, in certain specimens of blood the sedimentation rate remains the same throughout the twenty-four-hour period, for example, in malignant tumors and Hodgkin's disease. This observation was found to be correct in 95 per cent of 112 cases of malignant tumors and 14 cases of Hodgkin's disease. In 100 normals and 460 nonmalignant cases the rate was found to decrease steadily over a twenty-four-hour period (Table I). In 1940 Feldman[2] repeated Koster's work. His study consisted of 176 cases, of which 118 were proved malignancies and 55 were pathologic conditions other than malignancy. The percentage of positive results in the malignant cases was 95.7 per cent, and of negative results was 94.6 per cent in the nonmalignant cases. The methods of Koster and Feldman differed only in that the former used a Westergren tube and the latter used a Wintrobe tube in determining the sedimentation rate.

TABLE I (After Koster)

| SEDIMENTATION RATE | DIRECT | 1 HR. | 2 HR. | 3 HR. | 4 HR. | 5 HR. | 6 HR. | 24 HR. |
|---|---|---|---|---|---|---|---|---|
| Carcinoma of stomach (positive test) | 17½ | 17 | 16½ | 17 | 17 | 19 | 20 | 17½ |
| Ulcerative colitis (negative test) | 30 | 27 | 25 | 26 | 22 | 16 | 14 | 3 |

The importance of further confirmation of such a relatively simple laboratory procedure seemed to us obvious.

## METHOD

Our technique was essentially the same as described by Feldman. Two cubic centimeters of 3.8 per cent sodium citrate were placed in a dry 10 c.c. syringe and 8 c.c. of venous blood were drawn up. This mixture was used as the pooled specimen with which sedimentation rates were done directly and after one, two, three, four, and twenty-four hours. Koster and Feldman did rates for six hours the first day. We found that after four hours the tendency of the curve was established. The blood was thoroughly shaken before each

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Received for publication, June 24, 1942.

new sedimentation rate was done so that the cells which had settled out could be thoroughly mixed with the serum. The tubes were read exactly one hour after filling. Wintrobe sedimentation tubes and a Wintrobe rack were used. Both the tube and pipettes were carefully cleaned and dried with alcohol and ether before use. The pooled specimen was placed in a refrigerator overnight, and the next day the temperature of the specimen was allowed to return to room temperature before the twenty-four-hour rate was done. Duplicate tests showed no greater variation than 2 to 3 mm. in the comparison of hourly sedimentation rates (Table II).

TABLE II

| SEDIMENTATION RATE | DIRECT | 1 HR. | 2 HR. | 3 HR. | 4 HR. | 24 HR. |
|---|---|---|---|---|---|---|
| First test | 13 | 12 | 7 | 7 | 6 | 4 |
| Second test | 11 | 10 | 8 | 8 | 7 | 4 |

According to Koster, the criteria for a positive test (malignancy) are as follows (Table III):

TABLE III (After Koster)

| SEDIMENTATION RATE | DIRECT | 1 HR. | 2 HR. | 3 HR. | 4 HR. | 5 HR. | 6 HR. | 24 HR. |
|---|---|---|---|---|---|---|---|---|
| Type 1 | 17½ | 17 | 16½ | 17 | 17 | 19½ | 20 | 17½ |
| Type 2 | 70 | 73 | 78 | 76 | 95 | 96 | 94 | 107 |
| Type 3 | 30 | 30 | 35 | 38 | 39 | 38 | 36 | 16 |

Type 1: The rate of sedimentation remains at the same level throughout the twenty-four hours, or it does not vary to more than 20 per cent of the initial value.

Type 2: The rate of sedimentation markedly increases above the initial value in the course of the estimation and does not decrease to below the initial value.

Type 3: After having risen to above the initial value, the rate of sedimentation again decreases gradually, often to far below the initial value.

## MATERIAL

The patients were selected from the adult medical and surgical wards of Beth Israel Hospital and from our private practice. All diagnoses of malignancy were confirmed either by roentgenogram, biopsy, surgery, or necropsy. In the majority of instances the cases were taken as consecutive admissions. In all, there were 100 patients of whom 33 had proved malignancies.

## RESULTS

Our percentage accuracy for diagnosing malignancies was 54 per cent (Table IV) as compared with Koster's 95 per cent and Feldman's 95.7 per cent (Table V). Our percentage accuracy in nonmalignant cases was 72 per cent as compared with Koster's 100 per cent and Feldman's 94.6 per cent. Sedimentation tests were repeated on the majority of our false negatives two or more times.

## DISCUSSION

Our results obviously do not agree with those of either Koster or Feldman. In our series of malignant cases about one-half gave false negatives. False

negatives may be attributed to hemolysis of the specimen, insufficient shaking before each sedimentation rate so that the cells and serum are not thoroughly mixed, or not allowing the twenty-four-hour specimen to come to room temperature after removing it from the refrigerator. All of these make for a slower sedimentation rate and, therefore, tend toward the negative curve. These pitfalls were carefully avoided, as shown by the fact that ten patients on whom tests were done at the same time from specimens taken by two injections showed no more than 2 to 3 mm. variation in the hourly specimens (Table II).

TABLE IV

| | TOTAL NO. | WITH POSITIVE SEDIMENTATION RATE | WITH NEGATIVE SEDIMENTATION RATE | PERCENTAGE ACCURACY |
|---|---|---|---|---|
| Proved malignancies | 33 | 18 | 15 | 54 |
| Nonmalignant | 67 | 49 | 18 | 72 |

TABLE V

| | % POSITIVE | % NEGATIVE |
|---|---|---|
| Koster | 95 | 100 |
| Feldman | 95.7 | 94.6 |
| Hertz and Rinzler | 54 | 72 |

According to Koster and Feldman, false positives may result from the following drugs: the sulfonamides, potassium iodide, bismuth, salvarsan, aminopyrine. We have eliminated from this series any patients who were taking any of the sulfonamides. Other drugs taken by our patients include: atropine, phenobarbital, thyroid extract, thiamine chloride, caffeine citrate, ammonium chloride, amytal, cevitamic acid, feosol, and Lugol's solution. These drugs were taken in therapeutic doses by both the true negatives and false positives, both groups being nonmalignant. Diagnoses on some of the false positives were: chronic diffuse glomerular nephritis (3 tests); calculus of the common duct (5 tests); congestive heart failure (2 tests); and lupus erythematosis (2 tests).

We, therefore, feel that this test is of no value in our experience in accurately differentiating malignant from nonmalignant disease.

## SUMMARY

Sedimentation tests done serially according to the methods of Koster and Feldman on 33 patients with proved malignancies and 67 patients with pathologic conditions other than malignancy showed 54 per cent positive results in the malignant cases and 72 per cent negative results in the nonmalignant cases. We must conclude that in our hands the test is not accurate for use in the differential diagnosis between malignant and nonmalignant conditions.

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# MOUSE HOLDERS FACILITATING INTRAVENOUS AND INTRAPERITONEAL INJECTIONS*

ROY SCHNEITER, M.S., AND LAWRENCE R. CRISP

ALTHOUGH the intravenous injection of mice is a relatively simple procedure, the use of this route of inoculation is often limited because of the lack of a satisfactory apparatus for holding the animal during injection.

Very few satisfactory mouse or rat holders have ever been described. Burdon[1] described a mouse holder which consists essentially of a slitted, illuminated box on top of which is attached a wire cage for holding the animal. This holder, although it has many desirable features, appears to be somewhat cumbersome, and it is not adjustable to the length of the animal.

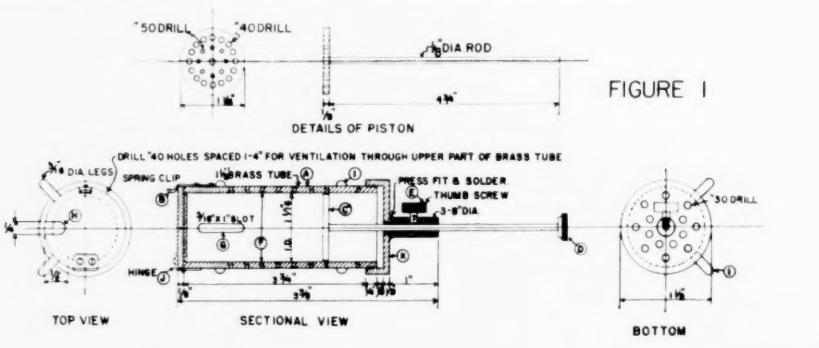


FIGURE 1

The apparatus described in this report was designed for simplicity and rapidity of operation and is adjustable to the length of the animal. Two types of holders have been designed (Figs. 1 and 2). The holders illustrated are made of brass tubing of suitable diameter for accommodating either large or small mice. The tube has a hinged, slotted door at the end or is hinged at the long, horizontal axis of the tube to permit the admission of the animal. A plunger for adjusting the length of the tube to the length of the animal is provided, and the animal is thus immobilized for either injection or bleeding. These holders are compact and easy to use and may be completely dismantled for cleaning and sterilization. With very little practice, mice may be readily induced to enter the holder.

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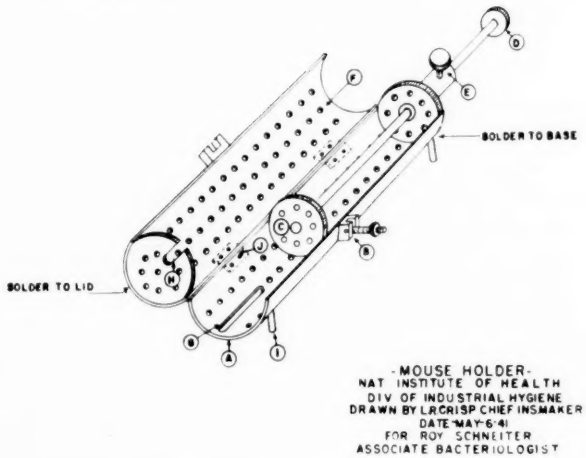
Received for publication, May 23, 1942.

By a selection of brass tubing of suitable diameter, either type of holder may be constructed for work with rats.

Each type of holder has been designed with a slit to permit intraperitoneal injections. A single worker with the aid of these holders can readily make intravenous or intraperitoneal injections without assistance.

FIGURE 2




Diagrammatic sketches of the two types of holders are given in Figs. 1 and 2. The essential parts of the holders are described as follows: *A*, Brass tube 5 inches long, 1⅛ inches outside diameter, and 1/16 inch wall; *B*, spring or swinging latches; *C*, plunger for adjusting holder to the length of the mouse; *D*, handle and rod for control of plunger; *E*, set screw for locking plunger rod after adjustment; *F*, air holes Nos. 30, 40, 44, and 50 drills; *G*, slit for intraperitoneal injections; *H*, slot in end of tube for accommodating tail of animal; *I*, legs for support of holder; *J*, hinges; and *K*, threaded cap for cleaning the end-opening holder.

In making intravenous injections the holders may be placed on the laboratory table top or mounted in a ring stand. The animal is placed in the holder, and the plunger is adjusted to restrict movement. Tail veins may be brought into sharp relief by the application of xylol or alcohol. One-fourth, 0.5, or 1 c.c. hypodermic syringes fitted with 1 inch, No. 26 gauge needles are the most suitable for intravenous injections. The needle is most easily inserted at an angle almost parallel to the tail. We have found 0.25 c.c. is the maximum quantity of inoculum which can ordinarily be injected into the tail vein of the average mouse.

## SUMMARY

Two types of holders have been described which facilitate the intravenous and intraperitoneal injection of mice and rats. These holders permit the utilization of these techniques by the individual worker, thus increasing the potential usefulness of these laboratory animals.

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# SPUTUM CONCENTRATION FOR THE CULTURE OF TUBERCLE BACILLUS*

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DIRECT cultivation of tubercle bacillus has not yet become a routine procedure because of drawbacks in the manipulations and concentration of sputum.

The most widely used method is perhaps that with sulfuric acid. The sputum is mixed with equal amounts of 15 per cent sulfuric acid and then, is washed and centrifuged.

This method, however, has some drawbacks because it fails usually with dense and viscous sputa, containing large amounts of mucus and albumin. Large flakes of mucus escape contact with the sulfuric acid and finally cause contaminations of the cultures.

To obviate this difficulty, I have found the following method more satisfactory and also more reliable, because it produces a complete fluidification of the sputum, thus lessening the danger of contaminations.

The formula I use is a modified Jousset's liquid for inoscopy:

| | |
|---|---|
| Pepsin | 1 Gm. |
| Glycerin | 10 c.c. |
| Hydrochloric acid | 15 c.c. |
| Sodium fluoride | 1 Gm. |
| Distilled water | 1,000 c.c. |

Mix equal volumes of sputum and solution and leave the mixture for four hours in the incubator at 37° C., shaking occasionally to facilitate homogenization.

At the end of this period centrifuge, wash with sterile saline several times to remove the acid, and make cultures with the sediment upon Petragnani's or Loewenstein's media, or prepare for microscopic examination.

The results are definitely superior to the sulfuric acid method. The culture method is preferable to animal inoculation, because it is less expensive, more reliable, and more rapid.

This method can be applied satisfactorily also to urine containing blood or albumin.

*From the Topeka State Hospital.

# CHEMICAL

## THE NATURE OF THE BLOOD IODINE AND ITS DETERMINATION*

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### HISTORICAL ASPECTS

SINCE the modest six-page contribution by Courtois[1] in 1813 in which he announced "the discovery of a new substance in seaweed," the study of iodine and its place in animal physiology has assumed ever-increasing interest and importance. In 1900 Gley and Bourcet[2] established the presence of iodine in the circulating blood and published figures that agree very closely with those accepted as normal today. The extent of these studies was limited by the fact that they required a liter of blood to complete a single analysis. In 1920 Kendall[3] reported values of about 13 gammas per 100 c.c. for whole blood in a small series. The first series of any size and the first values attempting to correlate the blood iodine level with clinical disorders of the thyroid gland was published by Veil and Sturm[4] in 1925. They reported values of 13 to 17 gammas per 100 c.c. as normal, and they found the blood iodine level to be increased in Graves' disease where their values varied from 21 to 70 gammas per 100 c.c. Veil and Sturm extended von Fellenberg's concept, which was widely and perhaps uncritically accepted, that the iodine in the blood existed in at least two forms. One form, which they called "organic," was insoluble in cold alcohol, and another, soluble in cold alcohol, they called "inorganic." In normal blood they found an average of 9 gammas per 100 c.c. "inorganic" and 5 gammas per 100 c.c. "organic" iodine. In Graves' disease they found that the increase was almost entirely in the "organic" fraction. In 1929 Lunde, Closs, and Pedersen[5] objected to the use of cold alcohol to separate the two forms of the blood iodine because, as they said, the "inorganic" component could never be extracted completely with cold alcohol and that further washings always contained iodine. They, therefore, proposed the use of hot alcohol in a Soxhlet apparatus using four volumes of alcohol to one volume of blood and extracting once for four hours. By this method the authors greatly increased the alcohol soluble or "inorganic" fraction so that normally they found from 7 to 12 gammas per 100 c.c. as "inorganic" and only 1 to 4 gammas per 100 c.c. as "organic" or remaining in the residue. Ultrafiltration was used as a means of separating the organic and inorganic fractions by Leipert[6] in 1937 and Trevorrow[7] in 1939, but their results are in conflict. The former found that from 70 to 90 per cent of the serum iodine was ultrafiltrable, whereas the latter found that absolutely no iodine passed through the ultra-

*From the Laboratories and the Medical Service of Dr. Eli Moschcowitz, Mount Sinai Hospital, New York.
Received for publication, February 25, 1942.

filter. More recently, Salter[8] has resorted to protein precipitation by heat and dilute acid and differentiates between the iodine fixed to the coagulum and that which can be recovered in the filtrate. He refers to the former as "protein" iodine and to the latter as "inorganic." He admits, however, that his cannot be used if iodides have been administered and that his method gives false values for the "protein iodine" fraction if iodide is added to plasma in vitro. It is obvious that methods which fail to eliminate ingested iodide have markedly restricted application.

The purpose of this study is to demonstrate the absence of true inorganic iodine in normal blood and to show that all the normally occurring iodine is bound to the plasma proteins. In addition, a method (dialysis) is presented which permits the determination of "protein bound" or nondialyzable iodine in the presence of inorganic iodine.

## METHODS

The analyses were carried out by the Matthews[9] modification of the Leipert method with the following minor variations:

1. The final oxidation to iodate was carried out in a small volume, using bromine vapor after the solution had been rendered just acid to methyl orange with phosphoric acid. Bromine was removed by boiling.
2. N/1,000 thiosulfate was used instead of N/5,000.
3. Sulfite was not used in the receiver.

This method is accurate to 5 per cent in the range of these experiments and recoveries of added iodine compounds are complete.

Duplicate determinations were done in every case. If the results did not check, new sets of duplicate determinations were set up.

The blank for the method is less than 0.05 gamma iodine per determination.

Dialyses were carried out in cellophane casings made by the Visking Company, and the duration of each dialysis was seventy-two hours in running water. Tap water can be used for dialysis if suitable correction is made for the iodine content of the water which enters the sac during dialysis.

## EXPERIMENTAL

The entire question of "organic" and "inorganic" iodine in normal blood was reopened by Trevorrow, who reported that she could extract all the iodine in normal blood by ethyl alcohol. If this is true, it is obvious that there can be no distinction between "inorganic" and "organic" fractions based upon alcohol solubility. We have been able to confirm her studies and have found that all the blood iodine can be extracted with either hot or cold alcohol, provided enough of the solvent is used and the extraction is carried on long enough. The extractions with cold alcohol were carried out in stoppered flasks in a shaking machine, and those with hot alcohol were carried out in a flask under a reflux condenser and also in a Soxhlet apparatus adding the blood to the extraction thimble after the proteins had been precipitated by the alcohol.

It is clear from these experiments and the studies of Trevorrow that no reliance can be placed upon so-called "organic" fractions of blood iodine based upon alcohol solubility. It is probable that individual workers have

TABLE I

SOLUBILITY OF NORMAL BLOOD IODINE IN COLD AND BOILING ALCOHOL
Iodine Content of Residue (in gamma)

(In all experiments 50 c.c. of whole blood containing 1.8 gamma of iodine were extracted with 400 c.c. of alcohol and each extraction lasted twenty-four hours)

| | COLD ALCOHOL | BOILING ALCOHOL | SOXHLET APPARATUS |
|---|---|---|---|
| After one extraction | 0.8 | 0.6 | 0.5 |
| After two extractions | 0.4 | 0.3 | 0.2 |
| After three extractions | <0.1 | <0.1 | <0.1 |

TABLE II

BLOOD IODINE VALUES BEFORE AND AFTER DIALYSIS
(In gamma per 100 c.c.)

| SOURCE OF BLOOD | IODINE CONTENT BEFORE DIALYSIS (AVERAGE) | IODINE CONTENT AFTER DIALYSIS (AVERAGE) |
|---|---|---|
| Sheep's blood | | |
| Lot 1 | 3.2 | 3.4 |
| Lot 2 | 3.0 | 3.0 |
| Lot 3 | 2.0 | 2.0 |
| Lot 4 | 2.1 | 2.4 |
| Lot 5 | 3.4 | 3.0 |
| Lot 6 | 1.9 | 1.9 |
| Average | 2.6 | 2.6 |
| Human blood controls | | |
| 1 | 3.2 | 3.0 |
| 2 | 3.6 | 3.0 |
| 3 | 2.6 | 2.9 |
| 4 | 2.0 | 2.1 |
| 5 | 2.4 | 2.3 |
| 6 | 3.0 | 3.0 |
| 7 | 4.0 | 3.9 |
| 8 | 3.5 | 3.7 |
| 9 | 3.4 | 3.4 |
| 10 | 4.1 | 4.0 |
| Average | 3.2 | 3.1 |
| Human blood Graves' disease | | |
| 1 | 8.6 | 8.0 |
| 2 | 10.2 | 10.6 |
| 3 | 13.0 | 13.0 |
| 4 | 12.0 | 11.5 |
| 5 | 7.5 | 7.5 |
| 6 | 8.0 | 8.1 |
| 7 | 9.7 | 10.1 |
| 8 | 6.8 | 6.4 |
| Average | 9.6 | 9.4 |

TABLE III

IODINE CONTENT OF WASHED RED BLOOD CELLS

| VOLUME OF PACKED RED BLOOD CELLS (C.C.) | IODINE CONTENT IN GAMMA PER 100 C.C. |
|---|---|
| 5 | <0.1 |
| 10 | <0.1 |
| 15 | <0.1 |
| 25 | <0.1 |

obtained relatively constant fractions because they kept the conditions of their extractions constant and, therefore, extracted a fairly constant proportion of the total blood iodine. This may also explain the variations in the ratio of "inorganic" to "organic" iodine from 3 to 1, as reported by Lunde and Closs[5] (or even 5 to 1) to 2 to 3, as reported by Perkin.[10] It is significant that the authors who obtained the highest "inorganic" fractions used hot alcohol in their extraction procedures.

It was clear to us that we could not use alcohol as a solvent to obtain significant partition values for the blood iodine or to differentiate between normal circulating iodine and ingested iodine. It occurred to us, as it had to Gley and Bourcet forty years ago, that the normally circulating iodine might be bound to protein and, therefore, resistant to dialysis. This proved to be the case as is shown in Table II.

It can be seen from these experiments that all the iodine that circulates normally or in Graves' disease is in a form that resists dialysis and is, therefore, fixed to a large molecule, probably protein.

TABLE IV

DIALYSIS OF WHOLE HUMAN BLOOD TO WHICH POTASSIUM IODIDE HAS BEEN ADDED IN VITRO

(In each experiment 25 c.c. of whole blood containing 0.8 gamma of iodine was used)

| AMOUNT OF POTASSIUM IODIDE ADDED (γ) | TOTAL IODINE CONTENT AFTER 72 HOURS' DIALYSIS (γ) | AVERAGE CHANGE IN IODINE CONTENT (γ) |
|---|---|---|
| 5 | 0.9 | −0.2 |
| | 0.8 | |
| | 0.8 | |
| | 0.6 | |
| 10 | 0.8 | 0 |
| | 0.7 | |
| | 0.8 | |
| | 0.9 | |
| 50 | 1.0 | −0.2 |
| | 0.8 | |
| | 0.6 | |
| | 0.7 | |
| 100 | 0.8 | −0.2 |
| | 0.8 | |
| | 0.7 | |
| | 0.8 | |
| 1,000 | 1.1 | +0.2 |
| | 0.7 | |
| | 0.8 | |
| | 0.7 | |
| 5,000 | 0.7 | 0 |
| | 0.8 | |
| | 0.9 | |
| | 0.8 | |

## DISTRIBUTION OF BLOOD IODINE IN CELLS AND PLASMA

There is no agreement in the literature regarding the proportion of the normally circulating blood iodine that exists in the red blood cells as compared with the concentration in the plasma. In 1900 Gley and Bourcet said that the red blood cells contained no iodine whatsoever. In 1940 Salter[11] indicated that

he thought there was as much iodine in the cells as in the plasma; he stated that in a euthyroid person the total blood iodine was 6 gammas per 100 c.c., the plasma iodine was 6 gammas per 100 c.c., and the iodine contained in the red blood cells was also 6 gammas per 100 c.c. Such values imply equal iodine distribution between plasma and cells when the hematocrit reading is 50 per cent. Trevorrow stated that "whole blood and plasma showed a distribution of the total iodine in proportion to the water content of the plasma and cells." Klassen, Bierbaum, and Curtis[12] found that the red blood cells account for only 5 per cent of the blood iodine and even this must be viewed with some reserve because the methods used have an error as large as 5 per cent.

TABLE V

BLOOD IODINE VALUES

(Values expressed in gamma per 100 c.c. when one gram of potassium iodide is administered by mouth three times daily for three days to normal adults)

| CASE | CONTROL PERIOD | | AFTER INGESTION OF POTASSIUM IODIDE | |
|---|---|---|---|---|
| | TOTAL BLOOD IODINE | NONDIALYZABLE BLOOD IODINE | TOTAL BLOOD IODINE | NONDIALYZABLE BLOOD IODINE |
| 1 | 4.5 | 4.0 | 825 | 4.0 |
| 2 | 4.4 | 4.3 | 385 | 4.0 |
| 3 | 3.6 | 3.6 | 624 | 3.9 |
| 4 | 4.3 | 4.1 | 465 | 4.3 |
| 5 | 3.8 | 3.6 | 726 | 4.1 |
| 6 | 3.4 | 3.4 | 1,290 | 3.4 |
| Average | | 3.8 | | 3.9 |

TABLE VI

DIALYSIS OF WHOLE BLOOD TO WHICH THYROXINE AND l-DIIODOTYROSINE (IN AQUEOUS SOLUTION) HAVE BEEN ADDED

| VOLUME OF BLOOD (C.C.) | IODINE CONTENT OF ADDED THYROXINE (γ) | THYROXINE RENDERED NONDIALYZABLE (%) |
|---|---|---|
| 5 | 2 | 46 |
| 5 | 5 | 40 |
| 5 | 20 | 40 |
| 5 | 50 | 34 |
| 25 | 2 | 68 |
| 25 | 5 | 60 |
| 25 | 20 | 48 |
| 25 | 50 | 40 |
| VOLUME OF BLOOD (C.C.) | IODINE CONTENT OF ADDED DIIODOTYROSINE (γ) | DIIODOTYROSINE RENDERED NONDIALYZABLE (%) |
| 5 | 2 | 17 |
| 5 | 5 | 12 |
| 5 | 20 | 8 |
| 5 | 50 | 5 |
| 25 | 2 | 35 |
| 25 | 5 | 28 |
| 25 | 20 | 17 |
| 25 | 50 | 11 |

Our studies with red blood cells washed four times with iodine-free 0.9 per cent sodium chloride solution reveal only traces of iodine. In our opinion the red blood cells are free of any significant quantity of iodine detectable by present methods. This is easily understood because all the blood iodine is in the plasma in a nondiffusible form, and the surface of the red blood cells probably acts as a semipermeable membrane, preventing the iodized molecule from entering.

It remained for us to demonstrate that iodide added to blood in vitro or ingested in vivo could be differentiated from the normally circulating blood iodine. For this separation the procedure of dialysis again proved completely satisfactory.

The representative data in tables demonstrate that the procedure of dialysis is a satisfactory answer to the problem of differentiating between normally circulating blood iodine and iodine ingested in the form of iodide.

In the course of these studies it became evident that certain organic iodine compounds reacted in a manner very different from the behavior of the inorganic salts of iodine. When these compounds were added to whole blood, plasma, or other protein solutions, they could not be removed quantitatively by dialysis, although they are easily dialyzable in aqueous solution. Apparently new compounds were formed with the proteins and these compounds resisted dialysis. A large series of organic iodine compounds was studied using different protein solutions. The quantitative aspects of the reaction are not simple, but the proportion of the added iodine compound rendered nondialyzable varied with the protein, the organically bound iodine compound, and the relative amounts of each employed. The following substances behaved in a qualitatively similar fashion: thyroxine, diiodotyrosine, tetraiodophenolphthalein, diodrast, and hippuran.

## SUMMARY

1. All the normally circulating blood iodine can be extracted with ethyl alcohol if proper precautions are taken. The distinction between organic and inorganic blood iodine based upon alcohol solubility is without foundation, and significant quantities of "inorganic" iodine in normal blood exist only as an artifact of chemical manipulations.

2. Practically all the normally circulating blood iodine is in the plasma. The amount present in washed red blood cells is too low to be determined by present methods.

3. All the normally circulating blood iodine or that circulating in Graves' disease is in a form which resists dialysis and is probably bound to protein.

4. Dialysis removed from the blood all iodine added or ingested in the form of iodide and can be used to remove ingested iodide known to the observer or unsuspected by him.

5. Dialysis does not remove completely added or ingested iodine in the form of diiodotyrosine or thyroxine.

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Acknowledgment is gratefully made to Roche-Organon, Inc., for their generous aid in supplying the thyroxine and diiodotyrosine used in the basic studies.

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# UREA SPOT TEST FOR THE DETECTION OF NITROGEN RETENTION*

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In 1922 Weltmann and Barrenscheen[8] called attention to the yellow reaction of urea with Ehrlich's para-dimethylaminobenzaldehyde reagent and suggested its use as a simple clinical test for nitrogen retention. This test was recommended some years later by Hunt.[3] In 1934 Patterson[7] described a spot test based on Weltmann and Barrenscheen's color reaction. The test was performed by mixing a few drops of blood with an Ehrlich reagent containing trichloroacetic acid in a small test tube and pouring the mixture on filter paper. A yellowish coloration of the moist area around the dark brown protein precipitate in the center indicated a substantial increase of blood urea. Krieger in 1934[4] confirmed the reliability of the test and introduced color standards of picric acid on filter paper. However, Heggie[2] in 1938, after investigating Patterson's spot test with blood specimens of known urea concentration, arrived at the conclusion that the method is inadequate not only for high concentrations, but also for the most important clinical range of 50 to 100 mg. per 100 c.c. urea. The potential usefulness of such a test as an emergency bedside procedure, if reliable, stimulated the present investigation and led to a satisfactory modification of the original Patterson test.

We agree with Heggie in concluding that Patterson's urea spot test in its original form is not adequate because under the conditions of the test even a considerable elevation of blood urea cannot be detected with any degree of accuracy. As long as the spot on the filter paper is still wet, it is impossible to see a definite yellow coloration, except perhaps in very high concentrations. In the course of drying the yellow color becomes gradually more distinct, but so does the color of the reagent itself. It is difficult, therefore, to note whether the yellow color is due to the urea reaction or to the color of the reagent.

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Received for publication, May 22, 1942.

From these findings two immediate objectives of our investigation were clearly indicated:

1. Inasmuch as the yellow color reaction becomes distinct after drying, a method of drying had to be found which would give a clear end point.
2. The error due to the yellow color of the reagent had to be eliminated.

### MODIFICATION OF THE UREA SPOT TEST

1. *Drying.* Experiments showed that it is not necessary to dry the filter paper for many hours (as advocated by Patterson and Krieger). It may be dried conveniently over any source of heat, provided the heat is applied equally to the entire spot. It is essential not to have the spot in direct contact with the source of heat, but at some distance from it. A bridge or desk lamp may be used as the source of heat or, better still, the filter paper may be allowed to rest on a steam radiator for several minutes while the doctor or technician is free to engage in other work.

2. *Elimination of yellow color of the reagent.* On the assumption that the yellow color of the reagent might be due to various degrees of purity, different preparations of p-dimethylaminobenzaldehyde were examined.* The Eastman drug gives a slightly more intense, yellow coloration than the English preparation. Therefore, only the Eastman p-dimethylaminobenzaldehyde was used.

It was found, in addition, that the solvents and the method of preparation are of importance. For example, alcoholic solutions increase the yellow color of the reagent considerably. It was also important not to use an excess of the reagent. The reagent finally was prepared as follows:

| | |
|---|---|
| P-dimethylaminobenzaldehyde "Kodak" | 0.5 Gm. |
| Trichloroacetic acid | 100.0 Gm. |
| Distilled water up to | 100.0 c.c. |

The reagent should be kept for convenience in a dropper bottle.

The p-dimethylaminobenzaldehyde dissolves easily in a concentrated trichloroacetic acid, producing a slightly yellow color. A drop of this reagent on filter paper dried for ten to fifteen minutes on a hot surface gives a pinkish to grayish spot, contrasting satisfactorily with the yellow reaction of the urea. Only on prolonged heating or after the paper is kept for many hours does the spot of dry reagent turn yellow.

In order to eliminate further any possibility of error due to the coloration of the reagent, it was found expedient to place the blood to be tested in the middle of a wet spot of reagent instead of mixing blood and reagent before applying the mixture to filter paper. This technique is sufficient to precipitate most of the blood proteins in a small drop and allows a clear differentiation between the yellow zone surrounding the dark brown spot of blood proteins from the outer pinkish to grayish zone of reagent itself. In addition, two further advantages exist. There is no necessity of using a small test tube for

*These samples were marketed by the following firms: British Drug Houses Ltd., London. Eastman Kodak Co., Inc., Rochester, N. Y. Eimer & Amend, New York. Hartman-Leddon Co., Philadelphia.

We wish to express our appreciation to the Eastman Kodak Co. for giving us a sample of their preparation.

mixing blood and reagent, and the amount of blood necessary for the test is decreased from several large drops to a single small drop, making this method a true microtechnique.

A drop of reagent is dropped on a small piece of filter paper. The finger is punctured in the usual manner for obtaining blood counts, and a small drop is allowed to come into contact with the moist area of the paper. The paper is dried, as described before, and is read after several minutes. A small spot of dark brown coagulated blood protein is seen in the center surrounded by a narrow yellow zone produced by the urea reaction and a pinkish-gray outer zone caused by the color of the reagent itself. The test is improved greatly in accuracy by turning the paper over and reading the result on the opposite side.* The intensity of the color reaction may be graded from 0 to 4 plus. It is wise at first to obtain a control reaction with normal blood, but those who have worked with this test soon found that a control was unnecessary. If the test is used infrequently, it is well to use a control.

## INTERPRETATION OF THE TEST

The test as described is sensitive enough to show a weak yellowish zone in normal blood. Comparatively small increases of urea nitrogen up to 25 mg. per 100 c.c. (about 50 mg. per 100 c.c. urea) can be detected by comparison with the reaction of normal blood, provided the conditions of the test (amount of wet reagent, surface of blood in contact with liquid reagent, and equal heating of both spots) are kept reasonably constant. The test is not a quantitative one except very roughly. Twenty to 40 mg. per 100 c.c. of urea nitrogen produced a yellow color slightly more intense than normal; 40 to 60 mg. per 100 c.c. gives a moderately intense reaction; above 60 mg. per 100 c.c. the color is very intense.

The only compounds in blood which might interfere with the urea reaction are the sulfonamides. These dyes can be considered as derivatives of sulfanilic acid which was listed as an interfering substance with Ehrlich's reagent by one of the authors (Naumann, 1938). Monto,[5] and more recently, Churg and Lehr[1] described a quantitative determination of sulfonamides in blood based on the yellow color reaction with Ehrlich's reagent. The urea spot test as described may also be used for the detection of the sulfa drugs in blood if only a rough estimate is required. Further investigation along these lines is now under way. While conditions with marked nitrogen retention will as a rule not be those in which sulfonamide treatment may be expected, it is well to keep this possibility in mind in those cases where treatment prior to the test is unknown.

## SUMMARY

1. A simple urea spot test for the detection of nitrogen retention is described, based on the yellow color reaction of urea with Ehrlich's aldehyde reagent.

2. The test, requiring a specially prepared reagent, filter paper, and a heat source, can be completed in ten minutes.

3. The sensitivity of the test is sufficient to detect slight, moderate, and marked increase of blood urea.

*Noted by Dr. Kwasecvska.

4. The urea spot test may also be used for the detection and rough estimation of sulfonamides in blood which give a similar yellow color reaction. The possible interference of urea and sulfonamides with each other is pointed out.

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## THE TRYPTOPHAN TEST IN GENERAL PARESIS*

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WE BECAME interested in the tryptophan test in general paresis as a result of our experience with a case of this disease on the wards of the Morrisania City Hospital in which the reaction was positive. A further evaluation of this test in syphilis was then undertaken and it was performed on the spinal fluids of 133 paretic persons. Cases were classified as paresis in which positive blood and spinal serology and the mental picture of paresis were all present. Because of the clinical features and positive serology both in blood and spinal fluid, three cases which did not have the typical paretic gold curve were considered as cases of paresis. In addition, the spinal fluids of several cases of taboparesis, latent syphilis, meningovascular or cerebral syphilis, and nonneurological syphilis, were studied. As a control series the fluids of 102 cases of alcoholism, the major psychoses and general medical conditions were examined. Fluids from patients with a history of syphilis or with a positive serology were excluded from this control group.

The technique of the test employed was that described by Lichtenberg[19] and Aiello.[1] It is essential that the spinal fluid be clear; we found, as have other workers,[2, 4, 19, 28] that xanthochromic, hemorrhagic, and purulent fluids gave "false positive" reactions.

Table I indicates the distribution of our cases. One hundred and nine, or 82 per cent, of the 133 paretic fluids gave a positive reaction. In the control

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Received for publication, June 4, 1942.

series only two positive responses were obtained; one was in a case of cerebral arteriosclerosis and one in a mild rheumatic fever. We had three persons with multiple sclerosis with paretic gold curves who gave negative responses.

TABLE I

| DIAGNOSIS | TOTAL NO. CASES | GOLD CURVE | NO. CASES | TRYPTOPHAN | | PER CENT POSITIVE |
|---|---|---|---|---|---|---|
| | | | | POSITIVE | NEGATIVE | |
| Paresis | 133 | Paretic | 130 | 106 | 24 | |
| | | Flat | 1 | 1 | 0 | 82 |
| | | Meningitic | 2 | 2 | 0 | |
| Taboparesis | 3 | Paretic | 1 | 1 | 0 | |
| | | Low paretic | 1 | 1 | 0 | |
| | | Syphilitic | 1 | 1 | 0 | |
| Tabes | 4 | Syphilitic | 4 | 0 | 0 | 0 |
| Asymptomatic neurosyphilis | 11 | Flat | 11 | 0 | 11 | 0 |
| Psychosis with cerebral or meningovascular syphilis | 13 | Paretic | 2 | 2 | 0 | |
| | | Syphilitic | 4 | 0 | 4 | 46.1 |
| | | Flat | 7 | 3 | 4 | |
| Syphilis with positive blood and negative spinal serology | 11 | Flat | 11 | 0 | 11 | 0 |
| Controls | 102 | Flat | 102 | 2 | 100 | 2 |
| Multiple sclerosis | 3 | Paretic | 3 | 0 | 3 | 0 |
| Psychosis with organic brain disease | 1 | Syphilitic | 1 | 0 | 1 | 0 |

The positive response in the cerebrospinal fluid to the tryptophan test depends upon the presence of the amino acid with the same name. Riebeling[24] states that no tryptophan is found in the normal spinal fluid. This substance, according to Mouriz,[21] is normally present in the cerebrospinal fluid up to 1 mg. per 100 c.c. Jequier and Roten[17] by adding tryptophan to normal spinal fluids which gave no response to the test found that if there is less than 0.0001 Gm. of this substance in the testing fluid the result is negative. At this level a faint ring is seen. Brugi[7] found that one part of tryptophan to 10,000 parts of diluent resulted in a positive reaction.

Boltz[6] studied the spinal fluid by the test which bears his name. Grossman[13] and Harris[15] believed the test was due to cholesterol, although Boltz was uncertain as to the chemical basis for the reaction. Later authors, Riebeling, Blix and Backlin[3] and Walker and Sleeper,[31] state that this is a test for tryptophan. Table II indicates the results obtained by Boltz and other investigators employing his test or modification of it.

In the report of Steele and Nicole,[29] six of the positive cases had a syphilitic gold curve; the remainder, including the negative responses, were in cases with a paretic gold curve. They stated that 8 per cent of neurosyphilities give a positive result, although they are not true cases of paresis. Greenfield and Carmichael[11] were of the opinion that there was no relationship between changes in the colloidal gold curve and the test. All Thomas'[30] cases of paresis had a paretic gold curve.

TABLE II

| TEST | AUTHOR | CASES | POSITIVE | NEGATIVE | PER CENT POSITIVE |
|---|---|---|---|---|---|
| Boltz | Boltz[6] | 22 paresis | 22 | 0 | 100 |
| | | 2 taboparesis | 1 | 1 | 50 |
| | | 6 tabes | 0 | 6 | 0 |
| | | 10 psychosis with cerebral arteriosclerosis | 3 | 7 | 30 |
| | Grossman[13] | 28 paresis | 28 | 0 | 100 |
| | | 1 tabes | 0 | 1 | 0 |
| | | 31 other diseases | 0 | 31 | 0 |
| | Duncan[9] | 27 paresis | 27 | 0 | 100 |
| | | 127 other mental conditions | 121 | 6 | 94 |
| | Harris[15] | 92 paresis | 89 | 3 | 97 |
| | | 5 neurosyphilis | 2 | 3 | 40 |
| | | 83 other mental conditions | 1 | 82 | 1 |
| | Steele and Nicole[29] | 36 paresis | 35 | 1 | 97 |
| | Greenfield and Carmichael[11] | 16 paresis | 15 | 1 | 94 |
| | | 6 tabes | 0 | 6 | 0 |
| | | 4 cerebral syphilis | 0 | 4 | 0 |
| | Schreus and Willms[26] | 11 paresis or taboparesis | 11 | 0 | 100 |
| | | 24 tabes | 15 | 9 | 62 |
| | Thomas[30] | 33 paresis | 23 | 10 | 70 |
| | | 1 tabes | 0 | 1 | 0 |
| | Piotrowski[22] | 10 paresis | 6 | 4 | 60 |
| | | 5 tabes | 0 | 5 | 0 |
| | | 10 cerebral degenerative disorders | 7 | 3 | 70 |
| Riebeling | Riebeling[24] | 73 paresis | 68 | 5 | 93 |
| | | | TRACES | | |
| Kraus and Mezey colorimetric method | Kraus and Mezey[18] | 11 paresis | 5 | 5 | 1 |
| | | 3 taboparesis | 1 | 1 | 1 |
| | | 6 tabes | 2 | 1 | 3 |
| | | 7 cerebrospinal syphilis | 3 | 2 | 2 |
| | | 5 latent syphilis | 1 | 3 | 1 |
| | | 1 gumma brain | 0 | 1 | 0 |
| Aiello | Aiello[1] | 7 paresis | | 0 | 7 | 0 |
| | | 9 tabes | | 0 | 9 | 0 |
| | Spillane[28] | 8 paresis | | 0 | 8 | 0 |
| | | 4 cerebral syphilis | | 0 | 4 | 0 |
| | Baxter[2] | 3 paresis | | 0 | 3 | 0 |

A positive tryptophan test has been considered by many writers to be specific for tuberculous meningitis.[1, 5, 8, 12, 14, 19, 23, 25, 27, 28] Spillane,[28] accepting the work of Fildes and Knight,[10] on the ability of the tubercle bacilli to synthesize their own tryptophan in vitro on media lacking this amino acid, believes that a positive test is, therefore, due to the presence of the tubercle bacillus. Boltz and Grossman,[13] interested in this test in syphilis, felt that it was an index of the destruction by the *Treponema pallidum* upon the tissues of the central nervous system. Massazza[20] thought that it was due only to the breakdown of protein. Mouriz and Piotrowski[22] believed that tryptophan was

increased in processes in which there is destruction of cells in the central nervous system. A similar view is offered by Kraus and Mezey.[18] The last-mentioned authors obtained a positive tryptophan test only in cases where other pathologic findings were present in the fluid, especially an elevated colloidal gold curve. They were of the opinion that tryptophan, though it is not the sole factor, may be one of the agents responsible for the gold curve. In our series we obtained more positive tryptophan tests in syphilitic fluids with a paretic gold curve than without one. Though this relationship is not strictly parallel, we have found many more positive tryptophan tests in syphilitic fluid with a paretic gold curve than without one. This would explain its highest incidence in paresis. On the other hand, in cases with paretic gold curves but no syphilis, as in multiple sclerosis, the test has been negative. This convinces us that more than one factor is present in syphilitic spinal fluid to account for the colloidal gold curve and the tryptophan reaction. Perhaps the greater destruction of parenchymatous nerve tissue in paresis than in the other forms of syphilis results in a greater likelihood of the simultaneous production of the factors responsible for the tryptophan reaction and the colloidal gold curve.

Greenfield and Carmichael found negative results to the test even in cases with total protein up to 1 per cent. Baxter[2] reported negative tryptophan tests in fluids showing an increased cell count and positive Pandy. Brugi and Herbert[16] believed the results paralleled the increase in albumin content and obtained positive responses in fluids in which the albumin was greater than 30 mg. per 100 c.c. Steele and Nicole, and Duncan[9] found a close correlation between the globulin and the Boltz test, and in none of their cases was the result negative when the globulin was positive. Piotrowski's work disclosed no association between the globulin content and a positive Boltz reaction. Walker and Sleeper demonstrated a varying intensity of response with the amount of protein in the spinal fluid but stated that the relationship is not a direct one. In our series of the 18 cases in which the quantitative protein was 50 mg. per 100 c.c. or over, five with figures of 100, 50, 119, 50, and 110 mg. per 100 c.c., gave a negative tryptophan reaction. The remainder in this series were positive.

Kraus and Mezey included two malaria treated cases; one with paresis gave a negative test, the other with tabes gave a positive tryptophan test. Harris found in malaria-treated cases that, although the Wassermann reaction had in most instances become less strongly positive, the Boltz test showed no change. Steele and Nicole state that the Boltz test was altered in about half the malarial treated cases, if considerable alteration from the original paretic gold curve had occurred. Riebeling, on the other hand, thought that the results were more positive in persons with general paresis who were treated, than in those not treated. Three of the five with paresis, in our series, who had received malaria therapy six weeks to four years prior to the examination of the fluid, gave positive tryptophan reactions. We feel that our series of cases treated with malaria or chemotherapy is too small to warrant any definite conclusion in regard to the effect of the therapy on the tryptophan response.

Kraus and Mezey found a negative test in old standing neurosyphilis or in cases that had no signs of parenchymatous involvement. Schreus and Willms[26]

noted an increase in the reaction with the passing of time. They found the Boltz reaction positive in only 26 per cent of 37 cases of less than two years' duration, and in 56 per cent of 72 cases of more than two years' duration.

## CONCLUSIONS

1. A total of 109, or 82 per cent, of 133 paretic fluids gave a positive tryptophan response. It was positive in 3 (100 per cent) cases of taboparesis, negative in 4 (100 per cent) cases of tabes, and positive in 5 of 13 or 46 per cent of cases of psychosis with meningovascular or cerebral syphilis.

2. The test is most often positive in cases of syphilis with a paretic gold curve, but does not occur in other diseases as multiple sclerosis where a paretic gold curve but no syphilis is found.

3. The test is not necessarily dependent upon a quantitative increase in the total protein of the spinal fluid.

4. In a control series of 102 cases in which there was no positive serology or history of syphilis, it was positive in only 2 cases, in one of cerebral arteriosclerosis, and in one of rheumatic fever.

5. From the literature we have tabulated the results of the Boltz and Riebeling tests which are probably dependent upon the tryptophan in the spinal fluid.

6. The spinal fluid must be clear because hemorrhagic, xanthochromic, or purulent fluids give false positive reactions.

We thank A. Wolfson, B.S., chemist at the Morrisania City Hospital, for aid in the examination of the spinal fluids.

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# EFFECTS OF DESICCATION PROCEDURES ON THE CHEMICAL COMPOSITION OF FECES, URINE, AND MILK*

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THE art and science of dehydration have been practiced from the earliest times of mankind, for the sake of either concentration or preservation. In some types of chemical work it is desirable not only to concentrate and to preserve a given mixture or substance, but also to determine quantitatively the amount of water contained therein. The method of choice in the removal of water depends upon the completeness of desiccation desired and upon the chemical or physical alterations that may occur as the material is being dried.

Food and other biological products may be concentrated and preserved, or their water content may be studied by dehydrating with heat, at atmospheric or under reduced pressure. The loss in weight that takes place during the dehydration process, before constant weight is reached, is generally assumed to be due, primarily, to the vaporization of water. Drying at temperatures of 70° C. and upward to that of boiling water, however, may result in the volatilization of essential oils,[1] loss of nitrogen,[1-3] decomposition of fats and carbohyates[4-6] and, in some cases, the removal of water may be extremely time-

*From the Research Laboratory of the Children's Fund of Michigan, Detroit.
Some of the data included in this paper were presented before the Biological Division of the American Chemical Society, at the One-Hundredth Meeting of the Society, Detroit, Mich., September 9 to 13, 1940.
Received for publication, June 10, 1942.

consuming. After protein-containing substance has been dried to constant weight, the final product may still contain from 2 to 7 per cent water.[7, 8] The use of temperatures of 100° to 110° C. for dehydration may be accompanied not only by a loss of volatile substances but also by a gain in weight from oxidation of fats and carbohydrates.[1, 6]

Some of the difficulties in the removal of water may be obviated by drying at lower temperatures (50° to 70° C.) in an inert atmosphere of nitrogen or carbon dioxide under partial pressure, if special equipment is available for this purpose. If heating must be avoided, the classic method of drying at room temperature in a partial vacuum over a desiccant may be used—a method which is tedious, time-consuming, and impractical where large volumes of water are to be removed in a short time. For rapid determination of the moisture content of biological material, reflux distillation with xylene or toluene may be applied with a high degree of efficiency and accuracy.[9, 10] This latter method has no application in the preservation or concentration of foods for edible purposes, however, nor is it practical in connection with certain types of organic analyses.

In continuous metabolic balance experiments over many days, with several subjects, the number of specimens of foods, urine, and feces involved precludes chemical analysis of all samples immediately following collection. Indeed, under the usual circumstances of staff limitation, it is not practical even to attempt all the analyses concurrent with the collections. These conditions have constantly pointed out the need for procedures by which biological specimens may be preserved for future analysis in a state chemically equivalent to that existing at the time of collection. Increasing knowledge of physiologic processes and wider recognition of the great stability of biological substances in the dry state have emphasized the necessity for a more practical method of desiccation, for preservation with less danger of denaturation than the procedures previously mentioned.[11]

Although removal of water vapor from frozen specimens by means of a chemical desiccant was used at the beginning of the century, only recently has the cryochem process ("desiccation *in vacuo* from the frozen state by means of chemicals"[12]) been developed to a high degree of refinement. This process offers a rapid and safe procedure which permits the preservation of biological material in an undenatured form for long periods of time, provided the samples are properly stored. The purpose of this paper is to point out the advantages of the cryochem procedure in accumulating biological materials for future analysis, and to demonstrate how other methods of desiccation may alter the chemical composition of the same materials. The illustrations given portray the influence of different methods of dehydration upon distribution of free fatty acids and soaps in the feces and the total fat and nitrogen contents of feces, urine, and milk. The fecal fat excretion and its partition in the feces of normal children, determined on cryochem-desiccated feces, is reported in another paper.[13]

## FECAL FAT

Fecal material is much more conveniently handled and accurately sampled for analyses in the dry state, when it can be pulverized and thoroughly mixed.

In determining the total lipid content of feces, whether the wet or dried stool material is used is of no consequence as far as the results are concerned, since most methods depend on primary saponification of the material. However, in the fecal fat partition it has been claimed[16, 17] that analyses on dried stools are inaccurate and that the lipid partition should be carried out only on the wet material. Tidwell and Holt[18] have shown, in a comparison of the fat partitions on wet and dry fecal samples, that there was no significant alteration in the fecal fat distribution when feces were dried at room temperature in air. In handling a large number of fecal samples over a period of time, it is impractical to attempt desiccation of them at room temperature in air. The usual procedure has been to dry feces under alcohol in a forced draught oven, maintaining a temperature below 70° C.

TABLE I

EFFECT OF DRYING METHOD UPON FAT COMPOSITION OF FECES

| CONSTITUENT* | METHOD OF DRYING SAMPLE | MG. PER GM. DRIED FECES | | CRITICAL RATIO | ANALYSIS OF VARIANCE | |
|---|---|---|---|---|---|---|
| | | MEAN ± $SE_M$ | SD ± $SE_{SD}$ | | F | $F^1$ |
| Free fatty acid | Oven | 42.86 ± 2.03 | 7.60 ± 1.44 | 13.61 | 190 | 9.07 |
| | Cryochem | 14.28 ± 0.55 | 2.06 ± 0.39 | | | |
| Soap | Oven | 54.64 ± 6.10 | 22.81 ± 4.31 | 3.05 | 61 | 9.07 |
| | Cryochem | 81.86 ± 6.50 | 24.34 ± 4.60 | | | |
| Free fatty acid plus soap | Oven | 97.50 ± 6.52 | 24.39 ± 4.61 | 0.14 | 0.19 | 9.07 |
| | Cryochem | 96.14 ± 6.69 | 25.05 ± 4.73 | | | |

*The fat constituents were determined on the two types of dried preparation from each of 14 samples.

Corresponding samples of the feces from a group of normal children* were oven and cryochem dried. Table I compares the results of free fatty acid and soap analyses of the two types of material. The average amounts of free fatty acids per gram of dried feces were 43 mg. for the oven-dried samples and 14 mg. for the cryochem-dried; the average soap contents were 55 and 82 mg. per gram, respectively. That real differences existed for both the free fatty acid and soap determinations between the values obtained upon the samples prepared in the two ways, is shown by the critical ratios and the Analysis of Variance[15] results. The conclusion apparent from the results is that during the process of drying in the oven, a portion of the soaps was hydrolyzed. This seems evident, also, when the total free fatty acid plus soap contents of the feces dried by the two methods are compared. The average total for the oven-dried material is 97 mg. per gram and for the cryochem-dried feces it is 96 mg. No real difference was found between the free fatty acid plus soap analyses made on samples prepared in the two ways, indicating that while the total remained unchanged there was a real change in its components. From these results it seems clear that when the fecal fat partition is to be determined, care must be exercised in selecting the method of desiccation if the analyses are to be performed on dried material.

*The experimental studies with normal children were made possible by the cooperation of the Methodist Children's Village, Detroit.

NITROGEN

One of the determinations most frequently performed on biological material is analysis for nitrogen, from the results of which the protein content may be calculated. During standing and dehydrating, the organic constituents are most susceptible to loss of nitrogen.[3] The effect of the cryochem procedure of desiccation on nitrogen content is illustrated by analyses of feces, urine, and milk.

*Fecal Nitrogen.*—The averages of results of determination of the nitrogen content of the feces of children who were subjects of a continuous metabolic balance study over a period of fifty-five days[14] are given in Table II. Each fecal sample represented a five-day period, and similar samples from each of several children were carefully collected and preserved, after preparing in the following manner: (1) A sulfuric acid digest[14] was made of one portion of the fresh feces and the nitrogen determined. This value has served as a standard for comparison. (2) A portion of the fresh feces was dried down, under alcohol, in a forced draught, steam heated oven below 70° C. (3) Another portion of the fresh feces was dehydrated by the cryochem process.

TABLE II

EFFECT OF DRYING METHOD ON NITROGEN CONTENTS OF FECES AND URINE

| MATERIAL | METHOD OF PREPARING SAMPLE | NITROGEN* | | CRITICAL RATIOS | |
|---|---|---|---|---|---|
| | | MEAN ± $SE_M$ | SD ± $SE_{SD}$ | | |
| Feces† | Acid digest | 1380 ± 37 | 145 ± 26 | Acid digest:cryochem dried | 1.5 |
| | Cryochem dried | 1309 ± 31 | 120 ± 22 | Acid digest:oven dried | 5.3 |
| | Oven dried | 1125 ± 30 | 116 ± 21 | Oven dried:cryochem dried | 4.3 |
| Urine‡ | Fresh, untreated | 9.52 ± 0.12 | 1.08 ± 0.08 | Fresh:cryochem dried | 0.5 |
| | Cryochem dried | 9.60 ± 0.11 | 1.05 ± 0.08 | | |

*In milligrams per day for feces and milligrams per milliliter of fresh urine.
†From analyses of 15 samples.
‡From analyses of 88 samples.

From comparisons of the nitrogen contents of the preparations, drying in the oven caused a considerable loss of nitrogen, an average of 18 per cent of the nitrogen content of the acid digest of the fresh feces. The feces preserved by the cryochem procedure showed an average loss of only 5 per cent of the value determined for the acid digest, although the analyses were made two years after the samples had been dehydrated in the cryochem, and the loss was consistent for all samples. The children excreted in their feces, averages of 1,380, 1,309, and 1,125 mg. of nitrogen per day, as determined upon the acid digest, cryochem-dried, and oven-dried feces, respectively.

Statistical evaluation has been applied to the results obtained for the fecal nitrogen in 15 samples each of the acid digest, cryochem-dried, and oven-dried feces. The critical ratio (Table II) leaves some doubt as to the significance of the difference found between the nitrogen values for the acid digest and for the cryochem-dried samples, but the Analysis of Variance ($F = 51.68$; $F^1 = 8.86$) demonstrated a real difference. For the cryochem-prepared samples versus the oven-dried samples, the critical ratio (Table II) showed a real statistical difference, confirmed by the Analysis of Variance ($F = 89.67$; $F^1 = 8.86$). While the fresh samples contained slightly more nitrogen than the cryochem-

prepared samples, the latter had considerably greater nitrogen contents than the oven-dried samples. The real difference between the results obtained on the cryochem- and the oven-dried samples is evident from the values given in Table II and is confirmed by the critical ratio of 4.3.

Several explanations may be offered for the nitrogen loss shown by the cryochem-dried fecal material. Feces are known to contain approximately 40 per cent bacteria and volatile nitrogen compounds may already be present, or be formed even while the product is frozen. It is possible, also, that although the dried samples were stored in desiccators over desiccchlora, they may have absorbed some moisture when the glass-stoppered bottles were opened to remove samples for other determinations. Despite the small loss of nitrogen from the feces dehydrated by the cryochem process and preserved for two years, it appears safe to conclude that this method of drying offers the best method for desiccating various biological materials with a minimum of chemical alteration.

*Urinary Nitrogen.*—A comparison has been made between the nitrogen contents of 88 samples of urine, as determined upon fresh and upon cryochem-dried samples. From the average data for these series of analyses the nitrogen contents of the two preparations appear similar (Table II). The averages were 9.52 and 9.60 mg. of nitrogen per milliliter of urine in the fresh and dried samples, respectively; the ranges of variation shown by the standard deviations (SD) correspond closely, and the critical ratio is only 0.5.

*Milk Nitrogen.*—Five samples of fresh milk were secured at weekly intervals and analyzed for nitrogen by the Kjeldahl method.[14] Portions of the same samples were dried by the cryochem procedure and the nitrogen similarly was determined. The averages for nitrogen, determined upon the dried and fresh samples, were 509 and 506 mg. per 100 Gm. of milk, respectively, which represents a difference of only 0.5 per cent in the nitrogen contents.

It should be pointed out in this connection that in determining the energy value of such substances as milk and urine in the bomb calorimeter, other methods of drying make it necessary to determine the nitrogen loss in drying and make a correction for this loss in calculating the heat of combustion of the original material. The cryochem desiccation process offers a means of eliminating the additional determination of nitrogen on the dried material and the nitrogen loss correction in the energy calculation.

## SUMMARY

The removal of water from feces, urine, and milk by the cryochem process (dehydration *in vacuo* from the frozen state by means of chemicals) permits preservation of the dry material indefinitely in an undenatured form if stored under proper conditions. This method of dehydration has many advantages over oven-drying. Oven-drying at 70° C., and under, hydrolyzes the soaps in feces, causing exaggerated values for the free fatty acids and a reduction in the soaps, although the total free fatty acid plus soap values are the same for both methods of drying. The nitrogen contents of the cryochem samples of feces, urine, and milk approximate those of fresh specimens. In determining energy by the bomb calorimeter, the cryochem-dried material permits greater

accuracy in analyses and economy of time and materials, eliminating one nitrogen determination and correction for nitrogen loss in drying.

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# ON THE PURIFICATION OF GUM GHATTI*

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ALTHOUGH the use of Nessler's reagent dates back to 1856, it was apparently Chiles[1] in 1928, who first emphasized the great gain in stability of nesslerized solutions when gum arabic was present. By its inclusion he successfully nesslerized Kjeldahl digests containing concentrations of ammonium ion far greater than is otherwise possible. Such application of a suitable protective colloid was indicated, of course, once the Nessler reaction color was suspected of being a colloidal suspensoid. Prussian blue sols, present in a micro glucose method, were stabilized with gum ghatti by Folin[2] in 1929. Looney[3] in 1930 employed this same gum for his simple blood urea method by direct nesslerization. A year later Walters[4] applied it to other nesslerized solutions, claiming gum ghatti to be much superior to Chiles' gum arabic, of which it is a variety.

As would be expected, the natural gums are very variable substances, and most samples contain interfering compounds, which can cause reduction of the double iodide of Nessler's reagent with the separation of what seems to be mercurous compounds. When Nessler's reagent is added to a water solution of most natural gums, a greenish-yellow turbidity appears, and slow precipitation follows. Fortunately, the colored ammonia complex of the Nessler reaction is less easily reduced but still turbidity often develops. These reducing materials are doubtless responsible for the limited use of the gums as an aid to nesslerization and for disagreement as to its value. For example, Looney found gum ghatti to retard greatly the rapid turbidity that follows in almost all direct nesslerization urea methods, while Hawk and Andes[5] reported increased turbidity.

Several investigators have tried to remove the objectionable reducing substances in the gum solution with only partial success. Oxidation was an evident approach. Chiles mentioned using sodium peroxide but discarded it and simply mixed a portion of Nessler's reagent with a greater volume of crude gum solution and waited for it to clear. Doneen[6] suggested Chiles' method of preparation whenever the gum solution proved too crude. Folin,[2] in preparing gum ghatti solution for the iron-reduction glucose method cited above, oxidized with potassium permanganate. Other analysts, such as Walters, and Lieboff and Koppel,[7] have precipitated the gum from aqueous solution by strong alcohol. Most workers, however, including Looney, and Daly,[8] used, if any, a crude solution. Herzfeld,[9] and Nichols and Willits[10] employed gelatin which has a much lower gold number[11] than the gums. We have tried tragacanthin, the soluble portion of gum tragacanth,[12] without much satisfaction.

*From the Research Department of the Barnard Free Skin and Cancer Hospital, St. Louis.

Received for publication, June 15, 1942.

## EXPERIMENTAL

Although most samples of "soluble gum ghatti," or of gum arabic, include contaminations like woody fibers, earthy material, insoluble gum, and coloring matter, the impurities most objectionable from the view point of nesslerization are calcium, magnesium, and ammonium compounds, but especially the reducing substances.

The first problem is that of dissolving the gum. Some texts give directions for suspending the sample at the top of a cylinder of cold water where it requires days to disperse. Others lengthily stir the powdered gum with cold water, a method by which solutions up to 10 per cent are prepared, an unnecessary concentration, which is as viscous as syrup and cannot possibly be filtered or centrifuged. It is not only easier but better to prepare the solution hot, for it is thus somewhat less frothy and a little lower in reducing substances. Snell and Snell[13] recognized this, for while their directions call for a laborious dissolving of the gum in cold water, they then recommend heating the solution over a boiling brine bath "to destroy reducing enzymes."

We also attempted the destruction of the reducing substances by oxidizers, such as potassium mercuric iodide, triiodide, permanganate, hydrogen peroxide, etc., without much satisfaction. Permanganate, as used earlier by Folin, gives only partial improvement, and the deep brown oxide of manganese remains peptized in the gum solution.

The expedient of mixing some Nessler's reagent with a much greater volume of gum solution to clear out the reducing substances was found to have certain disadvantages. The alkali of the reagent seems at first to increase the reducing power of the gum solution, doubtless by hydrolysis and other reactions. Consequently, it is not practical to incorporate a small amount of protective gum solution with undiluted Nessler's reagent stock before use. Upon mixing the solutions, a very pronounced turbidity develops. After many days, the supernatant liquid is clear above a greenish or black residue of mercurous iodide or mercury. However, despite decomposition of the added Nessler's reagent and part of the gum, the final solution, though alkaline, is substantially lower in reducing substances. Unfortunately, use of such gum ghatti solution requires a special Nessler's reagent containing less alkali and more of the double iodide.

The alcohol precipitation technique is evidently based upon the assumption that the chief interfering substances are the salts associated with the gums. Addition of alcohol to an aqueous solution of a gum up to a concentration of 60 per cent or more[11] causes separation. Long contact of the precipitated gum with strong alcohol lowers its solubility in water. The sticky, demineralized mass is then dried, pulverized, and dissolved in water. This is a messy procedure and one which removes the salts but only a small portion of the reducing substances. One can prepare a better (but not good) gum ghatti solution by simply washing the powdered crude sample with hot alcohol, drying, dissolving in water to a 1 per cent solution, treating with permutit, and filtering.

True gums can also be precipitated from aqueous solution by basic lead acetate, but they are thereby denatured and are no longer water-soluble.

Turning then to the possibility of removing the interfering materials by adsorption, a variety of adsorbing agents was tried. Alumina gave fair results,

but this oxide is indicated[14] to attach pentosan gums. The clays, kaolin, and Lloyd's reagent were slightly effective, but they were so finely divided that it was almost impossible to separate the suspended particles. Silica in the form of kieselguhr had the same fault; ground silica gel did not, of course, but neither was it very active. The zeolite, permutit, besides removing calcium, magnesium, and ammonia, seemed to adsorb some coloring matter and reducing substances. However, adsorbtive carbons proved most satisfactory, especially purified bone charcoal, which we found can take out the greater portion of the reducing substances.

### SUGGESTED PROCEDURE

Bring the desired volume of distilled water to a boil, and then transfer the beaker to a steam bath. Add enough of a good grade of powdered gum ghatti (e.g., Coleman and Bell "gum ghatti, soluble") to make a 1 per cent solution, and heat with frequent stirring for about one-half to three-quarters hour, or until all soluble material appears to be dissolved. (Heating over a direct flame can char the gum.) While still hot, filter with suction through a couple thickness of closely-woven cloth pressed down snugly in a Büchner funnel with a disk of wire screen. (Only the grosser particles will be retained, but even the coarsest crepe filter paper would soon clog.) Return the gum solution to the steam bath, add about 6 to 8 Gm. of dry, acid-purified bone charcoal powder per 100 c.c., cover with a watch glass, and heat with occasional stirring for one-half to one hour. Oddly, after the charcoal treatment, the viscosity of the gum solution is apparently greatly reduced, as it is then much more readily filtrable (although Rakuzin[14] states that gum arabic at least is not adsorbed by charcoal). Filter while hot through a fast crepe paper (e.g., Reeve Angel 202) in the Büchner funnel. The filtrate may be grayish, due to fine particles of carbon. If so, change the filter paper to a retentive one (e.g., Munktell OA) and refilter. For each 100 c.c. of the cool filtrate, add about 3 to 4 Gm. of *good* permutit (e.g., "according to Folin"—or purify as described by Hawk and Bergeim[15]). Agitate gently for around ten minutes and filter with crepe paper and suction.

"Bone charcoal" as often sold may consist of a great deal of calcium compounds (including sulfides) and a little partly decomposed organic matter along with the porous carbon. Use of such material can render the gum solution more impure than before. If acid-treated bone charcoal is not available, an ordinary grade (e.g., Mallinckrodt No. 4396) must be purified, as described by Hawk and Bergeim.[15] Over 80 per cent of the "charcoal" may dissolve in the hot, dilute hydrochloric acid. To avoid prolonged washing, the residue can be removed from the Büchner funnel after a couple hot water rinsings, suspended in a little sodium bicarbonate solution (test with litmus), then refiltered and washed again in the funnel. It is naturally important that the charcoal be free of any strong acid. The final product may be used over if washed well with hot water and dried in the oven. The permutit may also be washed, repurified, and used again.

### ALTERNATIVE PROCEDURE

A method not quite as effective as the foregoing, but much more convenient, is as follows: Prepare the gum solution on the steam bath as outlined

but to only 0.5 per cent concentration. Filter hot through cloth as described previously, and add about 2 to 3 Gm. of a good alkali-free "activated" wood charcoal powder (e.g., "Activated Charcoal, Merck") per 100 c.c. Heat on the steam bath with frequent stirring for at least one-half hour. Filter hot with suction through crepe filter paper and then through a more retentive paper to catch the finer carbon particles. With wood charcoal these particles are smaller and lighter, and hence more difficult to remove. A lower concentration of the gum solution is necessary. The permutit treatment may often be omitted when wood charcoal is the adsorbing agent.

All the experiments outlined here were made by nesslerizing in the old Folin-Wu tubes in the presence of 1 c.c. of 1:1 sulfuric acid, and with the Koch-McMeekin reagent as prepared with mercuric oxide,[16] itself a rather stable reagent. As little as 5 c.c. of the purified 0.5 per cent gum ghatti will stabilize 1.5 mg. of nitrogen per 50 c.c. for hours, and 10 c.c. (or equivalent in 1 per cent) will serve for as high as 2.5 mg.

In adding the gum solution to the micro-Kjeldahl digest to be nesslerized, it is well to dilute that acid solution first to avoid any chance of possible hydrolysis of the pentosan gum.

Gum solutions will slightly lower the intensity of the Nessler color, doubtless due to the influence on particle size. They must naturally be added to the standard also.

As some molds grow readily in gum solutions, it is best to include a preservative. This must be one which causes no reduction when mixed with Nessler's reagent. Chloroform definitely gives marked reduction from contact with the alkali (formate?). Walters used water saturated with thymol, a good preservative but one which can cause a slight reduction. Toluol was found most satisfactory, only a few drops being required to prevent mold growth without refrigeration. (It is possible, by adding too much toluol and shaking very vigorously, to form a toluol emulsion.)

## SUMMARY

Reducing substances naturally present in most samples of gum ghatti will often cause turbidity of Nessler's reagent when that protective colloid is employed. The greater part of the interfering materials can be removed by treatment of the hot gum solution with acid-washed bone charcoal (or less well by wood charcoal) followed by permutit. These procedures are described.

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# A NEW MODIFICATION OF THE THUNBERG METHOD FOR THE DETERMINATION OF DEHYDROGENASES IN TISSUES*

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FOR the past two years we have carried out systematic studies on the effect of toxins and viruses on the dehydrogenase systems of tissues. The results of these investigations will be published in a separate paper. The classical method for this purpose, that described by Thunberg, requires special tubes which must be thoroughly evacuated and shaken during the entire course of the experiment. It was soon felt that this was too cumbersome for large-scale experiments. A modification was designed, therefore, which, without apparent disadvantages, permits the performance of many tests in a relatively short period of time. Instead of using evacuated tubes, the reagents were mixed with melted agar and the reaction was observed in the solidified medium. By this device anaerobic conditions were obtained, and, at the same time, sedimentation of the tissues was prevented.† Since we feel that this method may have other applications, we are presenting the technique in some detail.

*Reagents.*—

I. 2 per cent agar containing 0.5 per cent dibasic sodium phosphate and adjusted to pH 7.4.

II. 0.002 M methylene blue in distilled water.

III. Solutions of the various substrates. Succinate was used in 5 per cent solution and the others, citrate, glycerophosphate, glucose, and lactate, in equimolar concentrations. The pH of all solutions was adjusted to 7.1 with sodium hydroxide.

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Received for publication, June 15, 1942.

†After the elaboration of this method, Isaacs and Nussbaum[1] reported another modication of the Thunberg method for the determination of the dehydrogenases of bacteria. They suspended resting bacteria in saline and excluded oxygen by overlayering the fluid with vaseline. In our earlier experiments we used mineral oil for the same purpose but did not find it suitable for tissues. Because of the constant shaking required to prevent sedimentation of the tissue elements, the continuity of the oil layer was disrupted and oxygen gained access to the underlying fluid. The use of agar, therefore, is an essential feature of our technique.

IV. Tissue suspensions. The organs were finely ground with sand and 10 per cent suspensions were made in saline.

### PROCEDURE

The agar was melted and 2.5 c.c. were put into each tube. When the agar had cooled to 42° C., 0.5 c.c. of methylene blue and 1 c.c. of substrate (both warmed to 37° C.) were added. This mixture was then added to 1.25 c.c. of tissue suspension, also previously warmed to 37° C. Complete admixture of the components were accomplished by rather vigorous rotation between the palms. Immediately afterwards the tubes were plunged into a freezing mixture of ice and salt in water and, when the agar had solidified, kept for an additional ten minutes in a refrigerator.

The tubes were then put into a water bath at 38° C. and observed for the decolorization of the methylene blue. If the tissues were ground finely enough, the time at which the end point of the reaction was reached could be determined accurately. Owing to some slight diffusion of oxygen from the air into the agar a narrow, well-demarcated zone of blue coloration was present at the surface but did not interfere with the reading of the results. In order to determine the degree of anaerobiasis in the agar, 1.25 c.c. of tissue suspension and 1 c.c. of a 1 per cent solution of paraphenylenediamine hydrochloride (neutral to litmus) were added to 2.5 c.c. of agar and handled as described above. A hazy gray color appeared in the agar indicating the presence of a trace of oxygen. This color, however, disappeared within a few seconds except for a well-defined, narrow black band at the surface. We may conclude from this observation that within a few seconds after the start of the experiment, the agar is free from oxygen. The quantitative relationship between the time required for decolorization and the amount of dehydrogenase will be discussed in a subsequent paper.

In order to test the reproducibility and consistency of the results, tests were done with the organs of two animals using succinate as a substrate. In both series each individual organ was tested five times under exactly the same experimental conditions. From the experimental data the arithmetic means and the standard deviations were calculated and are recorded in Table I.

TABLE I

DETERMINATION OF SUCCINASE ACTIVITY IN VARIOUS SAMPLES OF THE SAME ORGAN

| ORGAN | EXPERIMENT I | | EXPERIMENT II | |
|---|---|---|---|---|
| | MEAN* | STANDARD DEVIATION* | MEAN* | STANDARD DEVIATION* |
| Kidney | 19.8 | ± 0.68 | 9.1 | ± 0.26 |
| Liver | 18.8 | ± 0.68 | 10.4 | ± 0.20 |
| Heart | 16.0 | ± 0 | 10.9 | ± 0.90 |
| Brain | 28.4 | ± 3.4 | 30.2 | ± 1.99 |

*Time in minutes.

The values of the standard deviations show that the results in each experiment with kidney, liver, and heart are consistent. In the experiments with brain, the results vary more considerably, although the experimental conditions were kept strictly constant. This probably is due to the well-known chemical instability of brain suspensions.

It may be seen from these two experiments that there are large differences in the dehydrogenase contents of the organs of various animals. Table II shows the results of experiments with the organs of 20 animals. Again we confine ourselves to determinations of succinase.

TABLE II

DETERMINATION OF SUCCINASE ACTIVITY IN THE ORGANS OF 20 ANIMALS

| ORGAN | MEAN* | STANDARD DEVIATION* |
|---|---|---|
| Kidney | 19.2 | ± 4.13 |
| Liver | 21.7 | ± 5.53 |
| Heart | 24.9 | ± 6.08 |
| Brain | 46.8 | ± 15.47 |
| Adrenals | 36.8 | ± 8.72 |

*Time in minutes.

These results show that any comparison between normal and pathologic organs can be made only on a statistical basis.

SUMMARY

A new modification of the Thunberg method for the determination of dehydrogenases in tissues has been reported. Tissue suspensions, methylene blue, and substrates are mixed with melted agar and decolorization observed in the solidified medium. The agar provides anaerobic conditions and prevents sedimentation of the tissue elements. The simplicity of this method permits the carrying out of large-scale experiments in a short period of time.

REFERENCE

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# A PHOTOELECTRIC METHOD FOR THE DETERMINATION OF POTASSIUM IN BLOOD SERUM*

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THE growing importance of potassium in physiologic investigations is attested to by the multiplicity of methods proposed for its determination. This fact is also evidence that none of the methods so far used has received general acceptance as wholly satisfactory and reliable. In our studies on the metabolism of schizophrenic patients we have used many methods, but most of our results have been obtained by the silver cobaltinitrite method of Breh and Gaebler,[1] modified by the procedure of Taylor,[2] for handling the standard. We have not been entirely satisfied with this method as the degree of error was too high to allow the detection of small but significant changes. When the extinction coefficient for the color developed by this method on known potassium solutions was determined on different days, there was a marked divergence of values, especially at the higher levels. The results for different concentra-

*From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester.

Received for publication, June 22, 1942.

tions of potassium obtained at the same time followed Beer's law but were not consistent from day to day. Truzkowski and Zwemer[3] subjected the method to a critical analysis and recommended that the precipitation be carried out between 17° and 22° C. Robinson and Putnam,[4] however, claimed that greater sensitivity can be obtained by precipitating from a 30 volume per cent alcohol medium at 4° to 6° C.

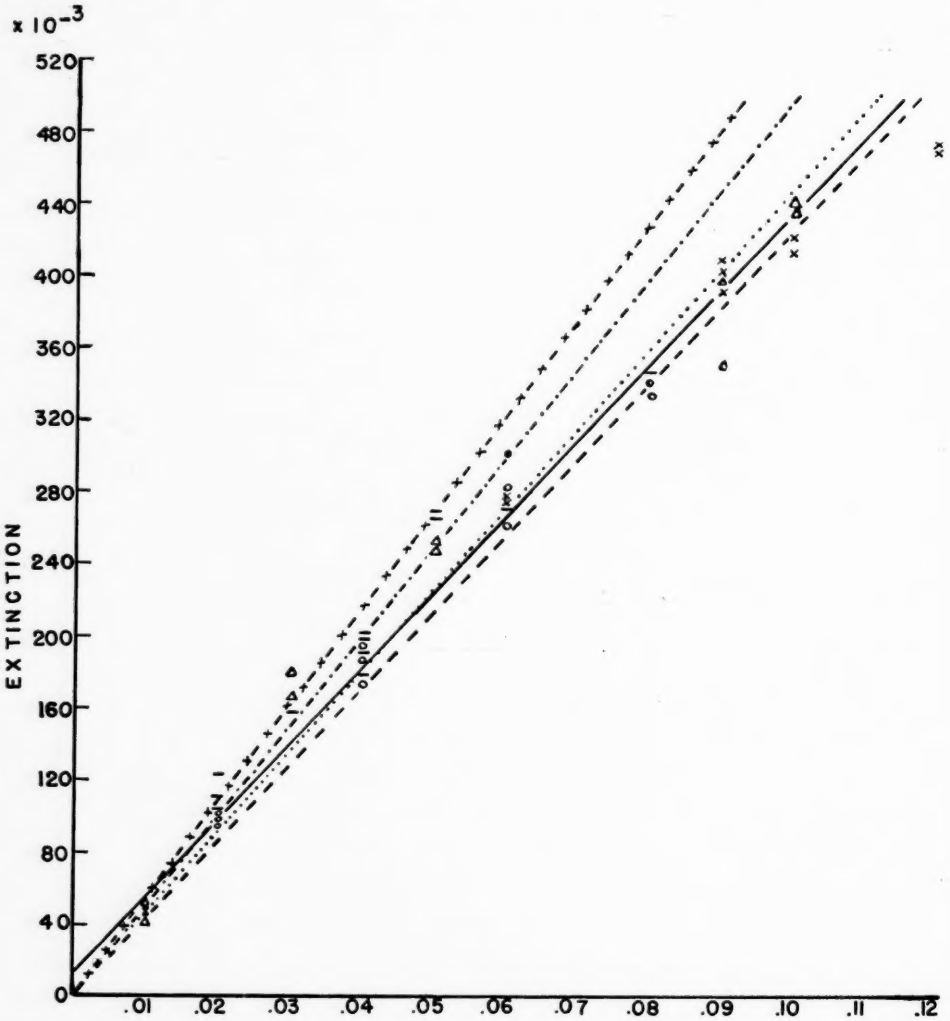


Fig. 1.—Variations in extinction produced by same amounts of potassium on different days with sulfanilic acid and α-naphthylamine. Temperature of precipitate held 20° to 23°. 1 c.c. of $AgNO_3$ = 20 c.c. of cobaltinitrite.

Harris[5] used a modification of the Breh and Gaebler method by precipitation from a 14 per cent alcoholic solution in a water bath at 20° C. for one-half hour. This period is much shorter than that recommended by most authors, and we have found it insufficient to obtain complete precipitation in very dilute solutions. Harris stated that the temperature could vary from 16° to 25° C. without change in the potassium/nitrite ratio. Our results indicate that the optimum range is between 18° and 20° C., since the potassium/nitrite ratio is

not constant above 23° C. and there is a tendency for fine needles of silver nitrite to precipitate below 16°. We have adopted his procedure of washing with an alcohol-ether-water mixture, since it is definitely superior in preventing flotation of the fine precipitate.

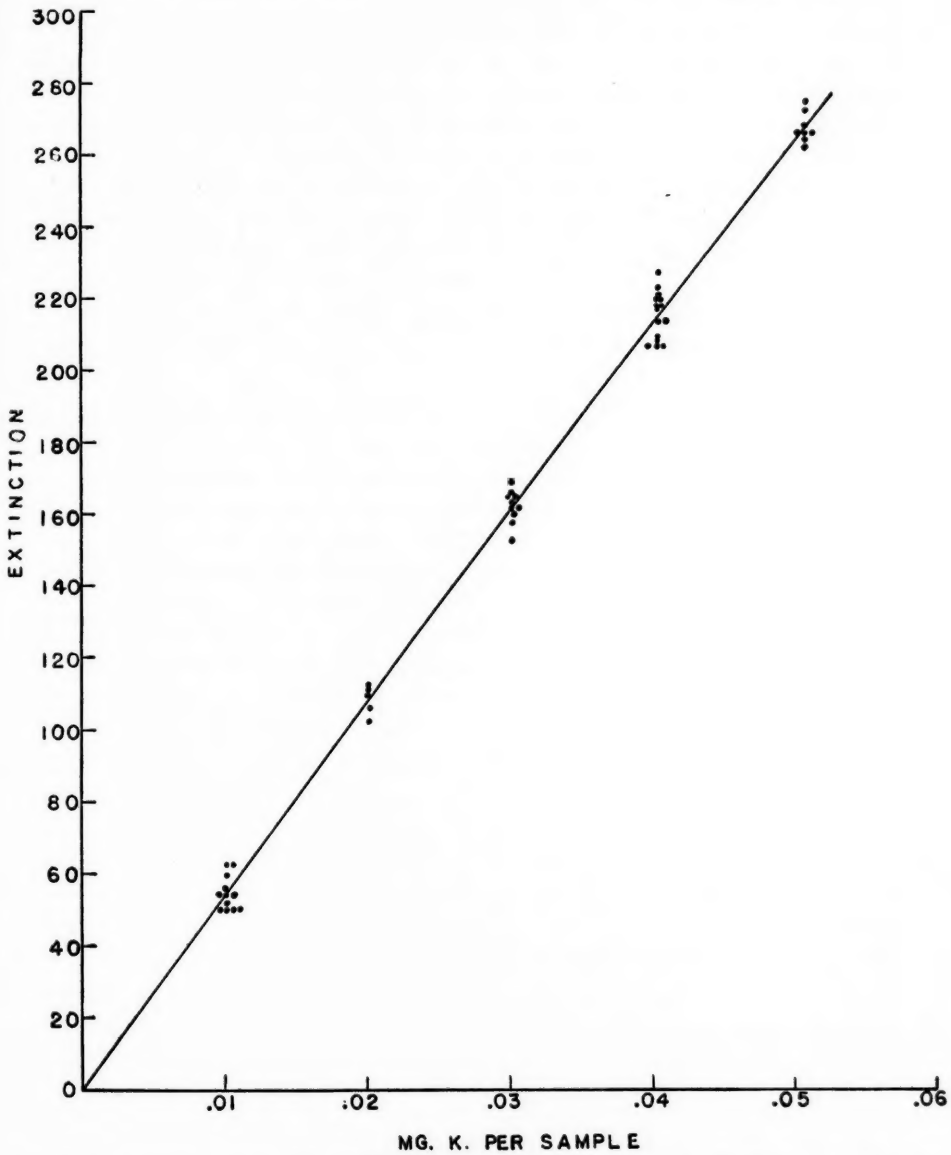


Fig. 2.—Extinction values for potassium silver cobaltinitrite with sulfanilamide and N-1 naphthylethylenediamine dihydrochloride.

The preparation of protein-free precipitates was also investigated. The commonly used Folin-Wu tungstic acid procedure plus silver nitrate was frequently followed by slightly cloudy filtrates which gave inconsistent results. The use of zinc sulfate and sodium hydroxide in combination with silver nitrate gave clear filtrates, but the results on known solutions were usually too high.

The use of sulfosalicylic acid plus silver nitrate was ruled out, because the results were high due to a reaction between the acid and the coupling agents used for the subsequent colorimetric procedure. Trichloroacetic acid could not be used, since all the samples that we tried gave turbid solutions with silver nitrate.

Weichselbaum, Somogyi, and Rusk[6] recommended the use of copper sulfate to replace the sulfuric acid in the preparation of filtrates. Our experience indicates that this method gives satisfactory results. They also recommended changes in the preparation of the silver cobaltinitrite reagent, but we were not able to obtain the results that they claimed for this modification and, therefore, returned to the use of the reagent of Breh and Gaebler.[1]

The chief difficulty in the use of a photoelectric colorimeter and a standard absorption curve arises from the variability in the amount of color produced by identical amounts of potassium on different days. This variability, as is shown in Fig. 1, arises from changes which occur in the solutions of sulfanilic acid and α-naphthylamine. Another disadvantage of these reagents is that the color produced is not stable and begins to fade after it attains its maximum development in about five to ten minutes.

It was felt that the substitution of the coupling reagent of Marshall[7] N-1 naphthylethylenediamine dihydrochloride for α-naphthylamine and sulfanilamide for sulfanilic acid might be advantageous. Shinn[8] has found these substitutions to give superior results in the determination of nitrite in foods, water, and sewage. We have been able to confirm her findings with regard to the permanence of the color produced by the Marshall reagents. Whereas the sulfanilic acid and α-naphthylamine showed a fading from an absorption reading of 77.2 per cent to 70.8 per cent on standing for twelve hours, the same amount of nitrite with the Marshall reagents showed a decrease in color only from 82.5 per cent to 81.8 per cent. The intensity of color was also considerably greater with the latter reagents. Although Shinn stated that the reagents would give satisfactory results for one month, we have found that values cannot be relied upon after a week when using the standard curve, and so we prepare these solutions fresh each week. If two standard solutions are run through at the same time as the unknown solutions, then satisfactory values can be computed from the line drawn through these two points for solutions kept up to a month. It should be noted, however, that the line must pass through the origin, and if this does not occur, some error has been made and new standards must be run.

TABLE I

| Mg. of K per 100 ml. final volume | 0.002 | 0.004 | 0.006 | 0.008 | 0.010 | 0.012 | 0.016 | 0.018 | 0.020 | 0.024 |
|---|---|---|---|---|---|---|---|---|---|---|
| No. of samples | 13 | 5 | 11 | 14 | 8 | 7 | 6 | 5 | 4 | 3 |
| Mean extinction | 0.054 | 0.108 | 0.162 | 0.216 | 0.270 | 0.276 | 0.338 | 0.399 | 0.428 | 0.471 |
| K | 22.5 | 22.5 | 22.5 | 22.5 | 22.5 | 19.0 | 17.5 | 18.0 | 17.5 | 16.0 |

## PROCEDURE

All glassware must be kept scrupulously clean. We have found it convenient to keep a set of glassware for use in potassium determinations only. The presence of ammonia will give falsely high results. Therefore, ammonia fumes must be avoided and tubes, must be capped at all times when not in manipulation. The silver concentration is also important, since an excess of

silver as well as low temperatures during the cobaltinitrite precipitation will give long needlelike crystals of silver nitrite which interfere with the results. For this reason, we have reduced the silver content of Breh and Gaebler's reagent by one-half.

A. *Precipitation of Protein and Chloride.*

1. To a clean test tube, add
   0.5 c.c. of serum,
   7.0 c.c. of distilled water,
   1.0 c.c. of 1.5% sodium tungstate, and mix
   1.0 c.c. of 2.5% copper sulfate.
   Stopper and shake well. Then add
   0.5 c.c. of 2.5% silver nitrate.
   Restopper and shake again.
2. Let stand for fifteen to twenty minutes and filter through a Whatman No. 5 paper. The first portion of the filtrate should be refiltered through the same paper in order to be sure of an absolutely clear solution, since a cloudy filtrate apparently gives a low result.

B. *Precipitation of Potassium With Silver Cobaltinitrite.*

1. To a clean 15 c.c. graduated centrifuge tube, add
   3 c.c. of the protein chloride-free filtrate,
   1 c.c. of 95% alcohol, and
   1 c.c. of distilled water.
2. Place in a water bath at 18° to 22° C. for five minutes.
3. Add 2 c.c. of silver cobaltinitrite reagent, cap and mix (by tapping, not by inversion), and replace in the water bath for two hours.
4. Centrifuge for fifteen minutes at 2,800 r.p.m.

C. *Washing the Precipitate.*

1. With a capillary pipette remove the supernatant fluid to the 0.2 c.c. mark.
2. Wash with 7 c.c. of wash reagent, rinsing down the sides and disturbing the precipitate as little as possible. If the tube is slanted, the remaining 0.2 c.c. of fluid will mix with the wash solution.
3. Centrifuge for fifteen minutes, decant, invert, and drain over filter paper for five minutes. Wipe the mouth of the tube.
4. Repeat this washing and draining twice more.

(After this stage, and only at this point, the procedure may be interrupted and the tubes allowed to stand overnight if one drop of distilled water is added to each tube to prevent the precipitate from drying up.)

D. *Digestion.*

1. Add 10 c.c. of approximately 0.2 N sodium hydroxide, breaking up the precipitate if possible.
2. Heat in a boiling water bath for ten minutes. A black precipitate remains.
3. Cool, make up to 10 c.c. with water, stopper, and mix. Replace stopper with centrifuge cap and centrifuge for five to ten minutes.

TABLE II

| EXP. NO. AMOUNT SERUM USED | SERUM K (MG./100) | | MG. OF K ADDED 100 ML. | TOTAL K OBTAINED | | TOTAL CALCULATED | ERROR OF TOTAL (%) |
|---|---|---|---|---|---|---|---|
| | VALUES | AVG. | | VALUES | AVG. | | |
| 1 ½ c.c. serum | 20.78<br>20.46<br>20.56 | 20.60 | 4.0 | 23.56<br>23.56 | 23.56 | 24.60 | −4.2 |
| | | | 8.0 | 28.18<br>28.18 | 28.18 | 28.60 | −1.5 |
| | | | 12.0 | 30.88<br>31.12 | 31.00 | 32.60 | −4.6 |
| 2 ½ c.c. serum | 20.46<br>20.66 | 20.56 | 4.0 | 24.66<br>24.12 | 24.39 | 24.56 | −0.69 |
| | | | 8.0 | 28.66<br>28.18 | 28.42 | 28.56 | −0.49 |
| | | | 12.0 | 32.22<br>32.22 | 32.22 | 32.56 | −1.0 |
| 3 ½ c.c. serum | 22.00<br>22.56 | 22.28 | 4.0 | 25.12<br>26.00 | 25.52 | 26.56 | −2.8 |
| | | | 8.0 | 29.78<br>29.56 | 29.67 | 30.28 | −2.0 |
| | | | 12.0 | 32.78<br>32.78 | 32.78 | 34.28 | −4.3 |
| 4* ½ c.c. serum | 20.18 | 20.18 | 4.0 | 24.12<br>24.12 | 24.12 | 24.18 | −0.24 |
| | | | 8.0 | 27.22<br>27.78 | 27.50 | 28.18 | −2.4 |
| | | | 12.0 | 31.12 | 31.12 | 32.18 | −3.3 |
| 5† ½ c.c. serum | 21.44<br>19.66<br>20.00 | 20.36 | 4.0 | 25.00<br>24.56 | 24.78 | 24.36 | +1.7 |
| | | | 8.0 | 28.18<br>28.34<br>28.34 | 28.29 | 28.36 | −0.25 |
| | | | 12.0 | 32.00<br>32.12 | 32.06 | 32.36 | −0.92 |
| 6 ½ c.c. serum | 21.18<br>21.00 | 21.09 | 4.0 | 23.78<br>23.78<br>25.34 | 24.30 | 25.09 | −3.1 |
| | | | 8.0 | 29.44<br>29.88 | 29.66 | 29.09 | +1.9 |
| | | | 12.0 | 33.12<br>32.12<br>32.12 | 32.45 | 33.09 | −1.9 |
| 7 ½ c.c. serum | 22.18<br>22.44 | 22.31 | 4.0 | 26.12<br>26.22 | 26.17 | 26.31 | −0.53 |
| 8 ½ c.c. serum | 28.46<br>28.46<br>28.46 | 28.46 | 4.0 | 32.50<br>32.34<br>32.12 | 32.32 | 32.46 | −0.46 |
| 9 ⅔ c.c. serum | 19.25<br>19.37 | 19.31 | 3.0 | 21.50<br>21 75<br>21.85 | 21.70 | 22.31 | −1.9 |
| | | | 6.0 | 24.90<br>24.90 | 24.90 | 25.31 | −1.6 |
| | | | 9.0 | 27.75<br>27.60 | 27.68 | 28.31 | −2.2 |
| 10 ⅔ c.c. serum | 20.60<br>20.60<br>20.40 | 20.53 | 3.0 | 23.00<br>22.60<br>22.60 | 22.73 | 23.53 | −3.4 |
| | | | 6.0 | 26.35<br>26.40 | 26.38 | 26.53 | −0.56 |

*Patient jaundiced. Icteric index 10.6.
†Patient had dinitrophenol.

TABLE II—CONT'D

| EXP. NO. AMOUNT SERUM USED | SERUM K (MG./100) | | MG. OF K ADDED 100 ML. | TOTAL K OBTAINED | | TOTAL CALCULATED | ERROR OF TOTAL (%) |
|---|---|---|---|---|---|---|---|
| | VALUES | AVG. | | VALUES | AVG. | | |
| 11 | 20.40 | | | 23.55 | | | |
| ⅔ c.c. serum | 20.50 | 20.45 | 3.0 | 23.10 | 23.33 | 23.45 | −0.51 |
| | | | 6.0 | 25.25 | | | |
| | | | | 25.15 | 25.20 | 26.45 | −4.7 |
| | | | 9.0 | 28.0 | | | |
| | | | | 28.25 | 28.13 | 29.45 | −4.5 |
| 12 | 25.82 | | | 29.12 | | | |
| ½ c.c. serum | 25.66 | 25.74 | 4.0 | 28.66 | 28.87 | 29.74 | −2.9 |
| | | | | 28.87 | | | |
| | | | 8.0 | 33.22 | | | |
| | | | | 32.50 | 32.86 | 33.74 | −2.6 |
| 13 | 19.66 | | 4.0 | 23.34 | | | |
| ½ c.c. serum | 19.66 | 19.66 | | 23.76 | 23.55 | 23.66 | −0.46 |
| | | | 8.0 | 27.44 | | | |
| | | | | 27.76 | 27.60 | 27.66 | −0.21 |

E. *Colorimetry.*

1. To a 100 c.c. volumetric flask, add
   2 c.c. of supernatant fluid from D,
   5 c.c. of distilled water,
   1 c.c. of 50% hydrochloric acid, and
   2 c.c. of 0.5% sulfanilamide.
2. Mix and let stand for three minutes, then add 1 c.c. of 0.1% N-1 naphthylethylenediamine dihydrochloride.
3. Make to volume and read after five minutes.

F. *Reagents.*

1. 1.5% sodium tungstate.
2. 2.5% copper sulfate.
3. 2.5% silver nitrate.
4. 0.2 N (approx.) sodium hydroxide.
5. 40% silver nitrate.
6. Sodium Cobaltinitrite Solution.[5]
   A. Dissolve 25 Gm. of cobalt nitrate crystals in 50 c.c. of water and add 12.5 c.c. of glacial acetic acid.
   B. Dissolve 120 Gm. of sodium nitrite in 180 c.c. of water (must be potassium free). Add 210 c.c. of B to all of A, and aerate the solution under the hood until all the nitrous oxide fumes are driven off. Place in the refrigerator where it will keep for about a month. *Filter each time before using.*
7. Cobaltinitrite Reagent.
   To 20 c.c. of filtered sodium cobaltinitrite solution, add
   1 c.c. of 40% silver nitrate.
   Shake well and filter to remove trace of precipitate which is undissolved.
8. Sulfanilamide Reagent.
   0.5% sulfanilamide in 30% acetic acid. Must be prepared fresh weekly.

9. Coupler Reagent.
   0.1% N-1 naphthylethylenediamine dihydrochloride in 30% acetic acid. Must be prepared fresh weekly.
10. Wash Reagent.
   2 volumes alcohol.
   1 volume ether.
   2 volumes water.

A standard curve was established by carrying out the foregoing procedure on standard potassium sulfate solutions containing 0.01 to 0.12 mg. of potassium per sample. The extended readings were obtained with a Lange photoelectric colorimeter against a water blank using a green filter having maximum transmission at 5,200 A.U. The results for values up to 0.05 mg. per sample are plotted in Fig. 2. As only one-fifth of the digested cobaltinitrite is taken for the final colorimetric reading, the absolute value of the potassium determined ranges from 0.002 to 0.010 mg.

It will be noted that the line which passes through the mean value for each concentration passes through the origin and follows Beer's law without deviation. Above 0.05 mg. of potassium per original sample the curve falls off sharply. This may be seen in Table I where the extinction constant K was calculated according to the formula $K = \frac{E}{CT}$, where $E$ equals the extinction, C = the concentration of potassium in milligrams per 100 c.c. of final volume, and T = the thickness of the solution through which the light must pass, which, in our tubes, is 1.2 cm.

In the usual procedure the concentration of the potassium in the final solution of the standard is 0.012 mg. and the readings are made at a point at which the color does not follow Beer's law.

Our method, therefore, has been adjusted to give readings corresponding to 0.006 mg. of potassium in the final solution. This is in the region in which there is no deviation from Beer's law and allows determinations to be made on serums containing up to 35 mg. of potassium per 100 ml. without dilution. This would cover the entire range which might be encountered in ordinary clinical practice.

Having established our table of values for known potassium, a series of blood serums were run to which varying amounts of potassium were added. The recovery values may be seen in Table II. It is interesting to note that, although different serums were used in each experiment, there is no wide variation in the potassium value for the serum alone. Cases 8 and 12 had an undetermined amount of potassium added before the known amount was added, which accounts for the high initial values in these two cases.

It will be noted that the determinations made in multiplicate on the original serum show a mean difference from the average value of each serum of only 0.15 mg., and this value is elevated somewhat by the poor result obtained in Case 5 where a difference of 1.78 mg. between the highest and lowest readings was found. If we exclude this case, the maximum difference between the highest and lowest readings of any serum was 0.56 mg. The mean error in the recovery of the added potassium was 1.89 per cent.

SUMMARY

A modification of the silver cobaltinitrite method for the determination of serum potassium adapted for use with a photoelectric colorimeter is described. By the use of sulfanilamide and N-1 naphthylethylenediamine dihydrochloride to determine the concentration of nitrite in the potassium silver cobaltinitrite precipitate a stable color is produced which is sensitive to 0.002 mg. of potassium in 100 ml. of solution. The color produced follows Beer's law up to a concentration of 0.01 mg. of potassium in the final solution, which would correspond to a serum potassium value of 33.3 mg. per 100 ml.

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# THE MICRODETERMINATION OF CHLORIDES IN VISCOUS BIOLOGICAL FLUIDS AND ITS RELIABILITY*

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THE need for a simple, rapid, and reliable method for determining micro quantities of chloride in 0.1 ml. samples of biological fluids like blood and viscous mucus is widely recognized, but no procedure which satisfies all these requirements is as yet available. Some adaptation of the Volhard titration (e.g., the method of Van Slyke[16]) would seem to be eminently suitable for the purpose, were it not that the precision of the silver thiocyanate titration is so poor as to rule this technique out of consideration for quantities of chlorine as low as 100 $\gamma$. In spite of the popularity of the Wilson and Ball[19] modification of the Van Slyke method for quantities around 3 to 5 mg., occasional articles which call attention to its poor reliability are still being published (e.g., Schales and Schales[12]). The chief cause of this inadequacy is the transience of the end point, which results from the dissociation of the ferric thiocyanate into its colorless constituents, aided

*From the Laboratories of the Mount Sinai Hospital, New York City.
This work was supported in part by a grant from the Friedsam Foundation.
Received for publication, June 25, 1942.

by the conversion of precipitated silver chloride to silver thiocyanate in consequence of the greater insolubility of the latter. Rosanoff and Hill[10] reported that this difficulty in the Volhard titration can be overcome in part by filtering off the silver chloride before titrating the excess silver with thiocyanate, but scrutiny of their data leaves some doubt regarding the relative magnitudes of the errors with and without such filtration. In order to save the time and labor of filtration, Keys[5] adopted the technique of Fiske and Sokhey,[2] and centrifuged before titrating in order to remove the silver chloride precipitate by packing it at the bottom of the titration tube. However, Kolthoff[7] has pointed out that separation of the silver chloride entails the removal of silver-ion by adsorption, and Whitehorn[17] found no improvement whatever as a result of such filtration. These reports led to the use of various chemical agents for reducing the transience of the end point. Smirk[14] reported that acetone in conjunction with alcoholic thiocyanate sharpens the end point by decreasing the dissociation of the ferric thiocyanate, but this effect was not confirmed by Wilson and Ball.[19] The use of nitrobenzene for the same purpose was advocated by Caldwell and Moyer[1]; and Keys[6] employed ether to collect the silver precipitate at the liquid-liquid interface and thus to reduce back reactions in the water phase, according to the principle of Rothmund and Burgstaller.[11] Toluene and benzene have been recommended by Stschigol[15] for the same purpose, but none of these techniques has as yet been generally adopted.

The method of Wilson and Ball has been in use in this laboratory for many years, but we also have never been satisfied with its reliability. Multiple titrations on the same solution are often in very poor agreement, particularly when they are done on different days. The magnitude of these errors is well illustrated by the two series of data for a set of 24 single determinations on the same solution, performed over a period of eight weeks (experiment G 33-9). For the titration of the blanks (2 ml. of 0.2 N silver nitrate, titrated with about 0.5 N potassium thiocyanate) the standard deviation of the entire distribution (s) was 0.0017 meq.* and the mean (M) was 0.394 meq. For the corresponding "unknown" titrations (2 ml. of 0.2 N silver nitrate + 0.5 ml. of 0.1 N sodium chloride) $s = 0.0015$ meq. and $M = 0.344$ meq. Hence, for the 24 titers of the sodium chloride, alone, calculated by difference of blank and unknown, $M = 0.050$ meq. and $s = \sqrt{(0.0017)^2 + (0.0015)^2} = 0.0023$ meq., or 4.6 per cent of the mean (see Addenda, especially equations 4 and 7). Two other sets of similar data (G 33-7 and 8) were in statistically reasonable agreement with these values, and they are all well within the experience of other laboratories in the use of such modifications of the Volhard method. These results, therefore, afford a fair measure of the poor precision of this method as ordinarily carried out. Since these titers were not correlated with the date of titration, there is no reason for suspecting a progressive change in concentration of any of the reagents on standing, and this is supported by the identity of the mean titers for sodium chloride in all three sets of data. Attempts to correlate the error with color intensity of the end point or with individual operator have been unsuccessful.

*All titers and standard deviations derived therefrom are here reported in milliequivalents instead of milliliters of potassium thiocyanate. This is done to facilitate the comparison of observations on different unknowns, and of titrations with reagents of different concentrations or volumes.

The afore-mentioned titrations were all performed in the presence of nitrobenzene, since without this substance (or with ether or acetone) the end point is even less reliable than with it. According to Caldwell and Moyer, the efficacy of nitrobenzene depends on its formation of "an insoluble layer over the precipitate, so that the rate of solution of the silver chloride is reduced to such an extent that it does not interfere in the thiocyanate titration," but a perfectly clear supernatant fluid is not essential. In our experience the silver precipitate appears to be removed by suspension within the layer of nitrobenzene, and this process rarely occurs completely. The action of the nitrobenzene can be reversed by the addition of more silver nitrate after the end point has been reached, and this explains why the removal of precipitate occurs only as one approaches the end point where the silver ion concentration is low. In our efforts to reduce the variability of our results we noticed that the uniformity of the titers increased with the completeness of removal of the precipitated silver salts by the nitrobenzene. Furthermore, the completeness of separation of the precipitate from the aqueous layer seemed to increase with the duration and vigor of shaking after each addition of reagent. Caldwell and Moyer state that agitation for thirty to forty seconds is necessary in order to form large spongy flakes, but they apparently did not associate such formation with the retention of these flakes within the nitrobenzene layer. Accordingly, we performed four series of titrations (G 33-10, 11, 12, and 13) by essentially the same procedure as before, except that additional precautions were taken in respect to the vigor and duration of shaking. The results showed marked and consistent improvement over those obtained without this innovation in technique. Thus, a pooled estimate of s (see Addenda, equations 8, 9, and 10) from all 6 sets of data *without systematic agitation* ($N = 170$) is 0.0016 meq., whereas for the 8 such sets on blanks and sodium chloride solutions *with active shaking* ($N = 290$), s is 0.0004 meq. This indicates a fourfold improvement in the precision—a result which was confirmed by similar titrations on standard hydrochloric acid solutions and oxalated whole blood (G 33-10, 12, and 13).

Our experience with the shaking technique has been so consistently good that there can be no doubt regarding the reason for the improvement in reproducibility of titration; i.e., *the systematic removal of silver precipitate from suspension in the aqueous phase by means of vigorous shaking with nitrobenzene.* Nitrobenzene has not always been efficient in yielding good agreement among multiple titrations, but this has resulted merely from the lack of adequate shaking of the titration mixture. The influence of stirring upon the rate of fading of the end point and, therefore, upon the titer, in the absence of nitrobenzene, was pointed out by Short and Gellis,[13] but Whitehorn[18] subsequently reported identical results with mild and with vigorous stirring. In consequence, the importance of intensive agitation in the Volhard titration has been generally neglected (Keys[5] does stir the titration mixture, by means of a jet of air, but only to insure uniformity of titration rate). However, our own observations leave no doubt of its importance for optimum reproducibility of the end point, particularly in view of the large number of titrations used for the estimation of the precision measures.

We have, therefore, adapted this technique to the determination of minute quantities of chlorine (as low as 90γ), and our results have been entirely consistent with expectations based on our experiences with the larger quantities. In order that the procedure may be applied to biological liquids of high viscosity (like mucous secretions) without loss of precision, we have modified the usual pipetting technique—but this may be disregarded for fluids of low viscosity.

## PROCEDURE

*Reagents.*—Silver nitrate (about 0.2 normal, not standardized), sodium chloride (0.1 normal, standardized gravimetrically, $s_M = 0.0004$ N), potassium thiocyanate (about 0.01 normal, not standardized*), ferric alum (6 per cent), nitrobenzene, and concentrated nitric acid.

The procedure is as follows:

1. Pipette 0.1 ml. of silver nitrate into each of a series of pyrex test tubes, 200 mm. by 24 mm. (outside diameter); designate 2 of these as "blanks."

2. Into each of 2 other tubes containing silver nitrate pipette 0.1 ml. of sodium chloride solution; these tubes are designated "NaCl standards" and serve in effect for the standardization of the potassium thiocyanate reagent.

3. Into each of another set of 2 or 3 tubes containing silver nitrate, introduce 0.1 ml. of the unknown solution, using the same pipette† for unknowns as for sodium chloride standards. With viscous liquids rinse the pipette twice with nitric acid and once with water—in which case the sodium chloride standard must also be delivered with rinsing.

4. To each tube add 1 ml. of nitric acid, less whatever may have been employed for rinsing. Cover the tube with a small watch glass and digest the contents on a water bath until the liquid becomes clear; this usually requires from one to two hours. Blanks and controls need not be digested, since their titers are the same with and without heating. Solutions brought to this stage can be stored in the dark for several days, or in dim diffuse light for one day, without any appreciable difference in titer.

5. After removing the tubes from the steam bath, cool and add 0.5 ml. of ferric alum and 6 drops (about 0.2 ml.) of nitrobenzene to each.

6. Titrate with the thiocyanate solution, using a 10 ml. burette graduated in 0.02 ml. A burette tip consisting of a hypodermic needle fitted to a Luer adaptor and the adjustable drop control device previously described (Hollander[3]) permit the addition of small volumes of reagent (0.003 ml.) with a minimum of time and effort. Add the thiocyanate slowly with frequent vigorous shaking, until the precipitate conglomerates into large flakes and is taken up by the nitrobenzene at the bottom of the tube. Slight opalescence in the supernatant fluid, caused by droplets of nitrobenzene, does not interfere with the end point nor reduce the precision of the method. As the end point is approached, shake the titration mixture vigorously after the addition of each drop of thiocyanate, for it is of the utmost importance that the precipitate be taken up entirely by the nitrobenzene at this stage. The nitrobenzene layer is broken up by this

*This solution is prepared with merthiolate (1:25,000) and then filtered to prevent the growth of organisms.

†These pipettes need not be calibrated, for reasons which are given later in this section. To facilitate their manipulation, without sucking the viscous specimen up above the graduation mark, we employ a special mechanical manipulator described elsewhere (Hollander and Stein[4]).

procedure, but it re-forms each time into an opaque spheroidal mass. The end point is indicated by the first faint orange-red tint of the supernatant liquid; under these conditions it is invariably stable for ten minutes and usually for much longer.

7. The concentration of chloride in the unknown, $[Cl]_{unk}$, is calculated by means of equation (2), in which the symbols have the following meanings: v = volume in milliliters of thiocyanate reagent required for unknown, blank, or sodium chloride standard as indicated by the subscript; ml = volume in milliliters of sample pipetted out; $[Cl]_{NaCl}$ = chlorine concentration of the standard sodium chloride solution.

$$[Cl]_{unk} = [Cl]_{NaCl} \left(\frac{v_{blank} - v_{unk}}{v_{blank} - v_{NaCl}}\right)\left(\frac{ml_{NaCl}}{ml_{unk}}\right) \quad (1)$$

Since the same pipette is used for unknown and for standard solutions, the last factor on the right becomes unity. Then, representing $(v_{blank} - v_{unk})$ by $\Delta v_{unk}$ and $(v_{blank} - v_{NaCl})$ by $\Delta v_{NaCl}$, we have

$$[Cl]_{unk} = \Delta v_{unk} \frac{[Cl]_{NaCl}}{\Delta v_{NaCl}} \quad (2)$$

The expression $[Cl]_{NaCl}/\Delta v_{NaCl}$ is a constant factor for any set of reagents, and this greatly simplifies the calculations required for a long series of analyses.

Van Slyke applied a negative "empirical" correction to each titration reading, because an excess of 0.04 ml. of reagent was necessary to give a decided end point. Keys stated that any method which omits separation of the precipitate from the titrated fluid yields titers which are slightly too high; this error might also be obviated by a correction term similar to Van Slyke's. The titers which we obtained without the effective transfer of the silver precipitate to the nitrobenzene layer were significantly higher than the values obtained by means of vigorous shaking, and this is qualitatively in agreement with the observations of Keys. However, our method of calculation obviates the use of any such correction term, because it would occur twice in the calculation of each $\Delta$ v-value and, therefore, cancel out completely.

It will be noted also that the foregoing procedure obviates the independent macrostandardization of the potassium thiocyanate solution, since the same end is attained by titration of the sodium chloride standard. The constancy of concentration of the reagent over a period of several months is demonstrated by the small scatter (s) of the data for blanks and sodium chloride standards throughout any one series of titrations, and, therefore, the titrations of blank and sodium chloride standards need be done infrequently. In view of the trouble commonly encountered with deterioration of thiocyanate solutions, this constancy of its concentration is particularly noteworthy, and we ascribe it to the preparation of the reagent with merthiolate and filtration.

## RELIABILITY OF THE METHOD

The reliability of an analytical procedure may be evaluated from the point of view of its accuracy (i.e., agreement between estimated and actual values; correctness) or of its precision (i.e., agreement among different estimates of the same value; reproducibility). In the following discussion the reliability has been considered from both of these angles.

As a measure of the *precision* of the method we performed a set of determinations on each of several solutions: standard hydrochloric acid in different concentrations, gastric mucous secretion, stomach contents, and oxalated whole blood. The number of titrations comprising each series and the statistical data are presented in Table I. In all but one of these series, the individual titrations were performed at various times throughout an interval of five weeks or more. The quantities of chlorine contained in the 0.1 ml. samples of the unknowns varied from 91 $\gamma$ to 439 $\gamma$, corresponding to concentrations of 26 to 124 mN.

Now, in calculating the chlorine concentration of any solution, we actually employ three different titers: for blank, for sodium chloride standard, and for the unknown itself. For the first two of these, we ordinarily use mean values obtained from a large number of titrations (e.g., series 3a and 3b); for the unknown, however, we usually employ a mean of two, or at most three, titrations. Referring to equation (1), we see that $s_M$ for $[Cl]_{unk}$ must be a function of the standard error of all three of these averages—$v_{blank}$, $v_{NaCl}$, and $v_{unk}$. But, since N for the two former is large compared with N for the unknown the $s_M$ values for the former are negligible with respect to $s_M$ for $v_{unk}$ (see equation 5). Consequently, $s_M$ for a Cl determination in triplicate may be taken equal to $s_M$ for the titer of the unknown alone, and this is equal to $s/\sqrt{3}$, where s is the standard deviation of the titration itself. From the data in the table it appears that s varies from 0.00009 to 0.00016 meq., and that these values are not correlated with the mean titer. Hence, the most representative estimate of s for a titration is given by a pooled estimate* made from all 8 series, with a total of 81 individual titrations ($\underline{N}$); by equation (11) this possesses the value 0.00010 meq. Therefore, $s_M = s/\sqrt{3} = 0.00006$ meq., and this is the precision measure for a titration done by this method in triplicate.

It must be remembered that the standard deviation observed for any other set of determinations may differ from this population estimate by amounts within the usual limits of statistical variation. Because of the very small number of titrations employed for any one determination, these differences may be relatively large. Furthermore, a percentage evaluation of precision (by means of the coefficient of variation, V) will vary with the magnitude of M according to equation (6). Thus, for a determination on 100 $\gamma$ of chlorine (0.0028 meq.), V = 2.1 per cent, whereas for 0.5 mg. V is one-fifth of this, or 0.4 per cent. In general, therefore, the standard error in milliequivalents affords a more significant precision measure for an analytical method than does the coefficient of variation.

High viscosity of the unknown specimen does not affect the reliability of the method. This is shown by comparing s for determinations on mucus, blood, and stomach contents (series 6, 5, and 14) with the values for inorganic solutions (series 3a-3e). The good agreement among these statistics results in great measure from our practice of delivering the sample for analysis by rinsing the pipette and our precaution not to draw the sample up above the graduation in the first place.

*The chi-square test for homogeneity of the variances ($s^2$) justifies the taking of such a pooled estimate.

An estimate of the *accuracy* of this method can be obtained by comparing its results with those determined by a macromethod on the same solution. For this purpose, three solutions of hydrochloric acid were prepared from a carefully standardized stock solution, the concentration of which was 100 normal by titration with sodium hydroxide (which in turn had been standardized against potassium acid phthalate). A gravimetric determination of chlorine in this solution as silver chloride gave a value of 99.9 mN. Based on the mean of these

TABLE I

SUMMARY OF MICROCHLORIDE TITRATION DATA

| SERIES | SOLUTIONS TITRATED WITH KCNS (0.01 N) | NUMBER OF TITRATIONS | MEAN TITER* N | ±s✦ (MEQ.) | ESTIMATED Cl CONCENTRATION | AMOUNT OF Cl |
|---|---|---|---|---|---|---|
| | | N | meq. | meq. | mN | γ |
| 3a (blank) | 0.1 ml. $AgNO_3$ (0.2 N) | 24 | 0.0205 | 0.00009 | | |
| 3b (standard) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. NaCl (0.1 N) | 18 | 0.0105 | 0.00009 | | |
| ---- | 0.1 ml. NaCl (0.1 N) by difference | - | 0.0099 | | | 352 |
| 3c (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.1 N) | 6 | 0.0105 | 0.00012 | | |
| ---- | 0.1 ml. HCl (0.1 N) by difference | - | 0.0100 | | 99.8 | 354 |
| 3d (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.05 N) | 6 | 0.0154 | 0.00011 | | |
| ---- | 0.1 ml. HCl (0.05 N) by difference | - | 0.0050 | | 50.4 | 179 |
| 3e (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. HCl (0.025 N) | 6 | 0.0179 | 0.00016 | | |
| ---- | 0.1 ml. HCl (0.025 N) by difference | - | 0.0026 | | 25.8 | 91 |
| 6 (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. mucus† | 6 | 0.0081 | 0.00013 | | |
| ---- | 0.1 ml. mucus by difference | - | 0.0124 | | 123.9 | 439 |
| 5 (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. stomach contents‡ | 6 | 0.0111 | 0.00007 | | |
| ---- | 0.1 ml. stomach contents by difference | - | 0.0093 | | 93.4 | 331 |
| 14 (unknown) | 0.1 ml. $AgNO_3$ (0.2 N) + 0.1 ml. oxalated whole blood | 9 | 0.0126 | 0.00007 | | |
| ---- | 0.1 ml. oxalated whole blood by difference | | 0.0075 | | 75.1 | 266 |

*Mean amount of potassium thiocyanate used in each titration (in milliequivalents).

†The mucus specimen was collected from a Pavlov pouch in the absence of acid secretion. The sample was extremely viscous and was homogenized before pipetting.

‡This specimen was a sample of fasting contents taken from a dog by means of a Rehfuss tube.

✦s The standard deviation of a single titration.

two values, the three solutions contained the following concentrations and quantities of chlorine: 100.0 mN (354 γ), 50.0 mN (177 γ), and 25.0 mN (89 γ). Six microdeterminations were performed on each of these solutions. The results are summarized in Table I (series 3c, d, and e), and the estimated concentrations are 99.8 mN, 50.4 mN, and 25.8 mN, respectively. These data afford ample evidence of the accuracy of this method.

## SUMMARY

1. A considerable improvement in the Van Slyke method for the determination of chlorides in biological fluids can be effected by the addition of nitrobenzene to the titration mixture, as recommended by Caldwell and Moyer, provided the liquid be agitated in such a way as to keep the precipitated silver salts adsorbed to the nitrobenzene layer. The procedure so modified is described in detail.

2. The accuracy and the precision of the method are determined in the range of approximately 100 to 500 micrograms of chlorine (0.1 ml. specimens with a concentration range of 25 to 125 mN). The standard deviation of a single titration, based on a pooled estimate from 81 titrations, is 0.00010 meq., or 1 mN. For a determination done, say, in triplicate, the standard error is 0.00006 meq., or 0.6 mN.

3. This high precision applies to liquids of high viscosity like mucus secretion as well as to hydrochloric acid solutions, provided the samples taken for analysis are pipetted with the proper precautions.

## ADDENDA

1. Let

$A_i$ = any one of a series of titers,
$N$ = the number of titers in the series,
$M$ = the mean of this series.

Then, the standard deviation for the series (and hence for a single titration) is given by s in the following equation:

$$s^2 \text{ (or } s_A{}^2) = \frac{\Sigma(M - A_i)^2}{N} \tag{3}$$

For the performance of various "tests of significance," it is customary to adjust $s^2$ with the factor $N/(N-1)$, particularly when $N \lessapprox 25$, in order to obtain an estimate of the population variance from that for a sample. This yields:

$$s^2 = \frac{\Sigma(M - A_i)^2}{(N - 1)} \tag{4}$$

In the present work we are concerned only with a description of errors and not with tests of significance, but we have made this adjustment as a matter of routine. Then the standard deviation of the mean (i.e., the standard error) is given by $s_M$:

$$s_M{}^2 = \frac{s^2}{N} = \frac{\Sigma(M - A_i)^2}{N(N - 1)} \tag{5}$$

and the corresponding percentage value, the coefficient of variation, is given by

$$V = \frac{100 \times s_M}{M} \text{ per cent} \tag{6}$$

2. For a series of N values, each of which is the difference of two such titers $(A_i - B_i)$, e.g., on blank and unknown solution, respectively, we have

$$s^2{}_{(A-B)} = S_A{}^2 + s_B{}^2 \tag{7}$$

provided $N_A = N_B$ or that they differ by an amount which is negligible.

3. Given p series of data, each comprising $N_1$, $N_2$, --------, $N_p$ titrations, respectively, and each possessing a mean and standard deviation $(M_1 \pm s_1)$, $(M_2 \pm s_2)$, -------- $(M_p \pm s_p)$, respectively. Then, for a pool of all the data we can calculate the same statistics by the following equations:

$$N = \sum_{i=1}^{p} N_i \tag{8}$$

$$M = \frac{\sum_{i=1}^{p}(N_i \times M_i)}{N} \tag{9}$$

$$s^2 = \frac{\sum_{i=1}^{p}(N_i s_i{}^2) + \sum_{i=1}^{p}(N_i d_i{}^2)}{N} \tag{10}$$

where $d_i = M_i - M$. If the several $M_i$ values are in fairly good agreement, then $d_i$ is negligibly small, and we have

$$s^2 = \frac{\sum_{i=1}^{p}(N_i s_i^2)}{N} \qquad (11)$$

For a discussion of the application of statistical methods to problems of chemical analysis, the reader is referred to an article by Power.[8] For a more detailed and rigorous, though elementary, treatment of these methods see Rider.[9]

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# A SIMPLE AND RELIABLE METHOD FOR THE DETERMINATION OF METHYL ALCOHOL AND FORMALDEHYDE IN THE AIR*

C. F. Ackerbauer, B.S., and R. J. Lebowich, M.D., Gloversville, N. Y.

IN THE COURSE of an investigation upon the clinical effects of methylation in workers engaged in the tanning industry, it became necessary to determine accurately the percentage of formaldehyde and methyl alcohol vapors in the air. In this paper, the word "methylation" is used to indicate a direct union or substitution of the methyl radical in certain chemical compounds in the tissue cells and body fluids. Nearly all the chemical methods which have been proposed for their estimation have been based upon the principle of a solution of these gases in water or some organic solvent.

The method submitted was developed to substantiate the cause of possible injurious effects found clinically in workers in industries employing formaldehyde or its derivatives and methyl alcohol. In recent years the use of these products has increased tremendously due to their employment in the manufacture of plastics, disinfectants, adhesives, textiles, rubber, and paper.

A critical and exhaustive survey of the literature failed to reveal the existence of any technical method for the accurate estimation of formaldehyde and methyl alcohol in the air, the necessity of which is patent.

Zhitkova[2] in his article on the determination of poisonous vapors in the atmosphere, mentions a method for the detection of these gases. These procedures have been carefully followed, checked and discarded because they did not yield reproducible results with a high percentage of accuracy.

Wright[1] investigated numerous proposed tests for the determination of methanol depending upon its oxidation to formaldehyde. He states: "As oxidizing agents hydrogen peroxide, chromic acid, ammonium persulphate, hot copper spiral and potassium permanganate were tried, but all except potassium permanganate were rejected as unsatisfactory." These findings have been confirmed by us. Rosaniline, identical with Schiff's reagent modified, proved to be the most sensitive and stable reagent and yielded the most reliable and reproducible results. It was necessary to modify this method slightly in order to differentiate between formaldehyde vapors and those of methyl alcohol. The commercial use of formaldehyde fully embraces the use of methyl alcohol because, as manufactured at present, it contains 1 to 8 per cent of methyl alcohol, according to specifications, in order to prevent the possible polymerization of formaldehyde to paraformaldehyde.

Adaptation of the modified Schiff's reagent required the forced solution of formaldehyde and methyl alcohol vapors into an aqueous solution, eliminating other fumes which might cause interfering chemical reactions, thereby markedly invalidating the accuracy of the method. In the field, chlorine, sulfur dioxide,

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Received for publication, June 26, 1942.

formic and acetic acid fumes reacted with the reagents in the receivers, destroying the formaldehyde condensation violet color formed by the union of rosaniline and formaldehyde. Therefore, it was essential to develop an apparatus for the absorption of these gases from the air.

The method satisfied the following criteria: (1) Complete absorption of the vapors of formaldehyde and methyl alcohol present in a measured volume of air with their negligible condensation upon the walls of the vessels containing the absorbing fluids. (2) Regulation of their rate of diffusion and absorption. (3) Complete elimination of interfering and extraneous gases.

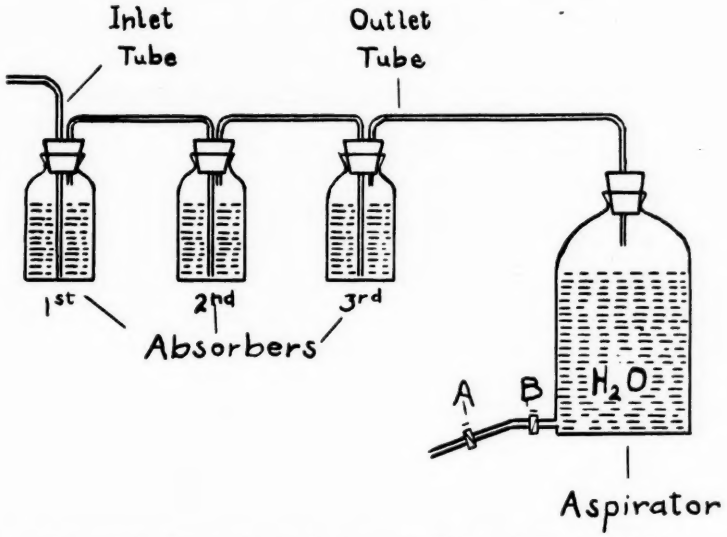



Fig. 1.—*A*, Screw clamp (snap). *B*, Screw clamp (variable).

## DESCRIPTION OF APPARATUS AND ABSORBING SOLUTIONS

For routine analysis an apparatus was constructed according to Fig. 1. The gas absorbers consist of three stock laboratory bottles fitted tightly with a two-holed rubber stopper. One hole of each stopper accommodates a ¼ inch diameter glass inlet tube extending to within ⅛ inch from the center of the bottom of the bottles. The other hole accommodates a ¼ inch diameter glass outlet tube which extends ¼ to ½ inch below the bottom of the stopper.

The bottles are so arranged in a series of three that the first inlet tube is joined to a glass tube of ½ inch diameter. The last outlet tube is connected to a 5 liter glass aspirating flask, the outlet of which regulates the flow and volume of the test sample of air through the system. Two screw clamps serve as valves to adjust the flow of water from the aspirator; one is permanently set to insure the same rate of flow for successive determinations; the other acts as a shut-off for the entire system. The diameters of the inlet openings are adjusted to a 1.6 mm. aperture to allow only small bubbles of the incoming vapors to pass through the liquids in the absorbers in a steadily flowing stream. Larger openings would permit the passage of excessively large bubbles carrying traces of impurities of sulfur dioxide, chlorine, and carbon dioxide, and these represent a source of considerable technical error. The use

of aerator stones was investigated and determinations with them proved inaccurate. They introduced a phenomenon of selective adsorption which did not remain constant throughout the period of time necessary to conduct a test.

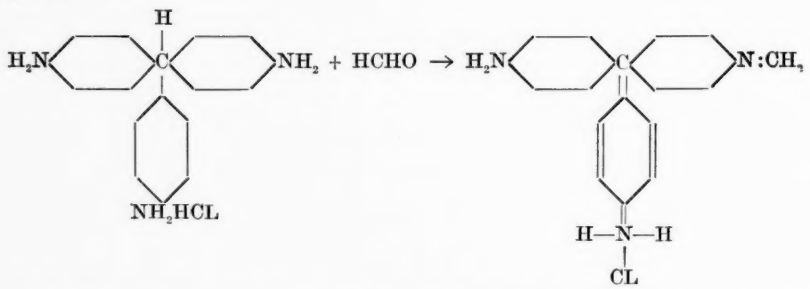
After many experiments the following solutions and their indicated concentrations were selected for the absorption of the interfering vapors and those to be estimated.

The first absorber containing 150 c.c. of a 1 per cent phosphoric acid and 2 per cent barium chloride solution in equal amounts removes all the sulfur dioxide fumes and the formic and acetic acid vapors. Frequent check tests were made on this solution to determine the amount of formaldehyde and methyl alcohol vapors absorbed, and the results revealed their presence only in minute negligible traces. This fact is believed to be due to the property of formaldehyde and methyl alcohol being more unstable in an acid solution than in an alkaline solution. The mild state of aeration tends to influence the presence of formaldehyde and methyl alcohol as a gas. The carbon dioxide, as it passes through water, forms carbonic acid, which reacts according to the following equation. The phosphoric acid acts as a carbonic anhydrase, as first purified by Meldrum and Roughton.[3] This accelerates the reaction in both directions: $H_2CO_3 + BaCl_2 \leftrightarrows 2HCl + BaCO_3\downarrow$.

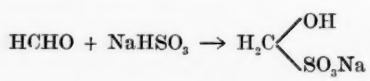
The second absorber contains 200 c.c. of an alkaline solution of 5 per cent potassium permanganate, which completely removes by oxidation all the methyl alcohol.

The third absorber contains 225 c.c. of modified Schiff's reagent, and the fourth, employed for experimental purposes, holds 200 c.c. 2 N sodium bisulfite solution.

The errors introduced by the entrainment of formaldehyde vapors into the first and second absorbers were found to be 1 part in 1,000 in the first absorber and 3 parts in 1,000 in the second absorber. In the third absorber, over 97 per cent of the formaldehyde vapors react with the modified Schiff's reagent, according to the following supposed equation forming the characteristic Schiff's violet color,[1] the intensity of which is proportional to the amount of reacting vapors.




The fourth experimental absorber, although unnecessary, was used to detect any escaping formaldehyde vapors by their reaction with sodium bisulfite solution, forming oxymethyl sodium sulfonate, according to the equation:



In some instances, volumes of vapors as high as 3.5 per cent were noted under conditions where the temperature of the surrounding atmosphere was above 80° F.

## DESCRIPTION AND RATIONAL OF METHOD

By means of the variable screw clamp, the vacuum created by the flow of water is regulated so that one liter of water flows freely without pulsations every twenty-five minutes. It is absolutely necessary that pulsations be eliminated, because many large bubbles may be forced spasmodically through the absorbers so rapidly that they may carry with them entrained vapors which are not absorbed in their respective solutions.

In order to absorb completely all the formaldehyde and methyl alcohol vapors, as well as to remove all interfering gaseous substances, it is necessary to allow at least 1 liter of water to flow from the aspirator. By experimentation with the object of reducing the percentage of technical error in the method to a minimum, it was found that the use of 5 liters of water yields very accurate results, but a volume of 5 to 10 liters is even more reliable and, therefore, desirable, as shown in Table II.

In a routine analysis, 1,000 c.c. of vapors are allowed to pass through the system in order to remove the air above the surface of the liquid in each absorber and saturate it with the vapors to be expelled from that solution. One liter of water is permitted to flow from the aspirator for a period of twenty-five minutes, and then its flow is stopped by the second snap clamp. The level of water is again adjusted in the aspirator and another 1,000 c.c. of air are inducted. While the aspirator is being filled, a vent must be opened or the liquids in the absorbers will be forced backward through the system, thus invalidating the results. This is repeated until at least 5,000 c.c. of air have been drawn through the receivers.

Absorbers 2 and 3 are removed, sealed with a rubber stopper, and are brought to the laboratory. To repeat the determinations, it is only necessary to renew the liquids in absorbers 2 and 3. The liquid in absorber 1 is sufficiently concentrated to be employed for at least ten determinations without renewal.

In the laboratory, the procedure for the analysis of the solutions in absorbers 2 and 3 is followed according to that outlined by Wright.[1] The potassium permanganate solution is decolorized with the oxalic-sulfuric acid solution and then the modified Schiff's reagent is added. Color comparisons are made with standards already set up and are matched with the naked eye, or preferably with a Hellige colorimeter.

Comparisons were made with the photoelectric colorimeter but were found to be unreliable, due to the inability of the photoelectric cell to differentiate between different wave lengths in the red and blue zones as well as to the intensity of these colors.

If the fourth absorber is used, the per cent of formaldehyde gas is determined volumetrically, using rosolic acid as an indicator, according to the equation:

$$HCHO + Na_2SO_3 + H_2O \rightarrow NaOH + NaSO_3 + (H_2COH)$$

In the field it was found that absorber 4 could be eliminated without seriously affecting the results.

Table II shows the results of the experiments conducted in a room, the known composition of its air being assumed to be 14 parts per 100,000 of HCHO and 0.6 parts per 100,000 $CH_3OH$. This assumption is based upon the fact that the possible condensation and adsorption of the vapors on the walls of floor and ceiling of the rooms and containers are negligible. Another assumption is that the rate of diffusion of these vapors becomes a constant after three to four hours. The tests show the effect of the rate of flow of 1 liter of air through the system in the period of time designated. All tests were conducted with the temperature maintained at 70° F.

TABLE I

| TEST | TIME (MINUTES) | ABSORBER 2 $CH_3OH$ (P.P.M.*) | ABSORBER 3 HCHO (P.P.M.) | ABSORBER 4 HCHO (P.P.M.) |
|---|---|---|---|---|
| 1 | 5 | Trace | Trace | 4 |
| 2 | 10 | Trace | Trace | 10 |
| 3 | 15 | Trace | 10 | 60 |
| 4 | 20 | Trace | 50 | 50 |
| 5 | 25 | 1 | 128 | 10 |
| 6† | 30 | 1 | 140 | 5 |

*Pulsations occur because the water did not flow freely but in drops.
†p.p.m. = parts per million.

TABLE II

| TEST | VOLUME IN LITERS | ABSORBER 2 (P.P.M.) | ABSORBER 3 (P.P.M.) |
|---|---|---|---|
| 1 | 1 | 1 | 130 |
| 2 | 3 | 1.5 | 132 |
| 3 | 5 | 5 | 140 |
| 4 | 10 | 7 | 148 |
| 5 | 12 | 6 | 150 |
| 6 | 15 | 7 | 149 |

Intervals of twenty-five minutes were decided upon for the period of the rate of flow. Table II shows the results of the experiments using the rate of flow of 1 liter of air for twenty-five minutes under the same previous existing conditions and with an increase of the volume of air passing through the absorbers before the laboratory tests are performed.

If the larger volumes of air are used, the intensity of color makes comparison with the standards more difficult to match with accuracy. It was also found that some vapors would escape through the aspirator, thus demonstrating that under these conditions the solutions in the absorbers had become saturated.

Table III shows the results of the experiments using the same standard conditions previously stated and varying the height of the inlet tube from the floor.

TABLE III

| TEST | HEIGHT OF INLET TUBE FROM FLOOR IN FEET | ABSORBER 2 (P.P.M.) | ABSORBER 3 (P.P.M.) |
|---|---|---|---|
| 1 | 1 | 20 | 100 |
| 2 | 3 | 8 | 180 |
| 3 | 5.5 | 3 | 160 |
| 4 | 7 | 1 | 80 |
| 5 | 10 | 1 | 30 |

A sealed room with temperature controlled at 70° F. was used for obtaining results shown in Table III. Vapors were introduced at a point as near the middle of the room as possible. This more closely resembles conditions as found in the field analysis. Although most of the material remain at the floor level, the height where the solutions have a greater tendency to vaporize would be around 5 feet.

These results show the effect of diffusion upon the vapors of $CH_3OH$ and HCHO in the air in a room after three to four hours. The height 5.5 feet was selected as the average height of a person present in a work room. The results of Table III also demonstrate the percentage of vapors in a room at this height where workmen might inhale or come into contact with these vapors.

## SUMMARY

A simple and reliable method is submitted for the estimation of methyl alcohol and formaldehyde vapors in the air, employing the modified Schiff's reagent of Wright, and having a possible technical error up to 7 per cent.

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# *REVIEWS*

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

## A Manual of Allergy*

THE author explains the writing of this book in the fact that while there are satisfactory large texts on the subject for the use of allergists, and while there are popular books through which the layman can understand allergy, there is need for an intermediate volume which will serve the medical man whose interest is only secondary and who does not wish to burden himself with extensive reading of the larger volumes.

Dr. Cohen's many important contributions to the subject give to his writings a generally recognized authoritativeness.

The book is well written and covers the subject excellently to the extent that general practitioners and nonmedical persons as well can gain a clear understanding of the processes of allergy. One is left with the feeling that Dr. Cohen would have done well to have enlarged it a little more with detailed discussion of the routines of specific diagnosis and treatment. One who has read the book and desires to proceed with allergic studies must still refer to the larger volumes. Although it would be desirable to have it all in one book, there is justification for the author's plan of presentation, in that those who actually wish to use allergic procedures should become acquainted with intricate details as well as with general principles. This certainly should make for better allergic practice.

In this sense we might consider the present volume as an Introduction to Allergy.

## An Atlas of Human Anatomy†

TOLDT'S *Atlas of Human Anatomy,* first published in this country in 1919, has gone through two editions and four printings. This no doubt accounts in part at least for the surprisingly reasonable cost of the latest printing. The volumes consist almost exclusively of plates, each of which contains duplicate Latin and English terminologies. Division into two volumes makes for easy handling. Although the sequence of plates is that commonly used in most anatomies, beginning with bones, joints, and muscles, and ending with the nervous system and special senses, the sequence continuity is more logical than in some other texts, facilitating progression from one area to a neighboring one. This is especially illustrated in the muscles of the lower extremities.

## Internal Medicine in Old Age‡

*INTERNAL MEDICINE IN OLD AGE* can be divided roughly into two parts: the first, a short survey of the nature and problems of aging and old age; the second, a comprehensive review of the clinical and diagnostic features and therapeutic management of those

*A Manual of Allergy—For General Practitioners. By Milton B. Cohen, M.D., Director of the Asthma, Hay Fever, and Allergy Foundation; Visiting Physician in Allergy, St. Alexis Hospital, Cleveland, Ohio. Cloth, 156 pages, $2.00. Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers, New York and London, 1941.

†An Atlas of Human Anatomy. For students and physicians. By Carl Toldt, M.D. Assisted by Professor Alois Dalla Rosa, M.D. Adapted to English and American and International Terminologies by M. Eden Paul, M.D. Brux., M.R.C.S., L.R.C.P. Vol. I. New second edition containing sections on Regions of Human Body, Osteology, Arthrology, Myology (Figures 1 to 640, and general index to the two volumes), 390 pages. Vol. II. New second edition containing sections on Splanchnology, Angeiology, Neurology, Organs of the Senses (Figures 641 to 1505, with appendixes and general index to the two valumes.) Cloth, copyright 1919. Reissued in two volumes, June, 1926. Reprinted January, 1941, The Macmillan Company, New York.

‡Internal Medicine in Old Age. By Albert Mueller-Deham, M.D., Associate Visiting Physician, Welfare Hospital for Chronic Disease (Second Division), Department of Hospitals, New York City; formerly Clinical Professor of Internal Medicine, University of Vienna Medical School, and Chief of Medical Division, Municipal Hospital for Chronic Disease, Vienna; and S. Milton Rabson, M.D., Assistant Professor of Pathology, New York Post-Graduate Medical School, Columbia University (on leave); Lieutenant Commander, United States Naval Reserve. Cloth, 396 pages, $5.00. A William Wood Book. The Williams & Wilkins Company, Baltimore, 1942.

diseases most common in the late decades of life, as well as of diseases more common in early years which present special problems in the aged.

The book should be of great value to those who desire an introduction to the field of geriatric medicine, as well as to those practicing physicians who desire ready reference to the pictures presented by disease in the aged. It is simply written and well-documented. The authors' practice of including a liberal number of thumbnail case histories adds interest to the reading and emphasis to the text, underlining the thesis that diagnosis of disease in the aged presents special problems, is not to be undertaken without mature reflection, and is, in spite of all, often mistaken or missed.

*Internal Medicine in Old Age* offers a wealth of therapeutic suggestions. Nothing seems omitted which might bring relief to the patient. There are valuable suggestions concerning nursing technique as well as chemotherapeutics. The reviewer recommends the book to student and practitioner alike as a valuable and timely review of geriatric medicine.

## Clinical Pellagra*

LIKE the period at the end of a paragraph this book punctuates an epoch in the study of pellagra. Dr. Harris starts his discussion with a comprehensive historical review of the early recognition and incidence of the disease, and the early theories as to its causation. This makes interesting reading. Next is a summary of the experimental investigations, those preceding vitamin studies and the vitamin studies themselves.

The major portion of the book is devoted to present knowledge of the etiology, pathology, symptomatology, diagnosis, prognosis, and treatment of the disease. The chapter on Prophylaxis is especially important.

Collaborating contributors from the several institutions, especially in the South, where constructive research has been carried out, have written chapters on the work done in these institutions.

Here we find in a single volume all that need be summarized concerning pellagra.

## Laboratory Diagnosis of Protozoan Diseases†

COLONEL CRAIG'S manual of laboratory methods for the diagnosis of diseases caused by protozoan organisms is especially appropriate at the present time when, with American forces in tropical climates, the problem of protozoan infection bids fair to become extremely important. Somewhat over a third of the book is devoted to amebic infection and considerable space is also given to malaria. The remainder of the book deals with the other common tropical infections, as well as protozoan infestations commonly seen in the United States, such as the flagellate infections.

The volume will undoubtedly be widely read.

## The Principles of Neurological Surgery‡

THE second edition of Davis' *Neurological Surgery*, like the first, contains full discussion not only of neurological procedure but also of neurologic diagnostic methods. For this reason, it will be useful both to surgeons and to neurologists and internists.

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*Clinical Pellagra. By Seale Harris, M.D., Professor Emeritus of Medicine, University of Alabama, Birmingham, Ala. Assisted by Seale Harris, Jr., M.D., formerly Assistant Professor of Medicine, Vanderbilt University, Birmingham, Ala.; with Foreword by E. V. McCollum, Ph.D., Sc.D., LL.D., Professor of Biochemistry, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore, Md. Cloth, illustrated, 494 pages. The C. V. Mosby Company, St. Louis, 1941.

†Laboratory Diagnosis of Protozoan Diseases. By Charles Franklin Craig, M.D., M.A. (Hon.), F.A.C.S., F.A.C.P., Colonel, United States Army Medical Corps. Retired D.S.M. Emeritus Professor of Tropical Medicine, Medical School, Tulane University; Member, American Academy of Tropical Medicine, American Society of Tropical Medicine, American Society of Parasitologists; Honorary Member, American Society of Clinical Pathologists, etc. Cloth, illustrated with 54 engravings and 4 colored plates, 349 pages, $4.50. Lea & Febiger, Philadelphia, 1942.

‡The Principles of Neurological Surgery. By Loyal Davis, M.S., M.D., Ph.D., D.Sc. (Hon.), Professor of Surgery and Chairman of the Division of Surgery, Northwestern University Medical School, Chicago, Ill. Cloth, ed. 2, thoroughly revised with 154 engravings and 298 illustrations and 5 colored plates, 503 pages, $7.00. Lea & Febiger, Philadelphia, 1942.

## Biological Symposia*

*BIOLOGICAL SYMPOSIA* comprises four symposia arranged by the Council of the American Society of Biological Chemists for the Chicago meeting of the Federation in April, 1941. The following subjects of current interest are discussed by authorities in each field:

End Products of Nitrogen Metabolism in Plants; End Products of Nitrogen Metabolism in Animals; Merging of Growth Factors and Vitamins; The Oxidation of Branched-Chain Fatty Acids; The Origin and Regulation of Ketone Bodies from Fats; The Anabolism and Function of the Phospholipids; The Formation of Animal Body Fat. Oxidation Catalysts; Phosphorylation of Glycogen and Glucose; Oxidoreductions in Carbohydrate Breakdown; Pyruvate Oxidation and the Citric Acid Cycle. Choline, the B Vitamins and Fat Metabolism; The Relation of Choline to the Kidneys; The Nature and Significance of Cholinesterase; Interrelationships Between Choline and Other Methylated Compounds.

## Textbook of Embryology†

NOT even embryology has reached a static stage in medical science. Jordan and Kindred's fourth edition has quite a number of changes from the third edition. New subjects include embryonic hormones, the *in vitro* development of mammalian embryos, transplantation, and extirpation experiments designed to reveal differential potencies of different portions of the blastoderm, results of administration of growth and sex hormones, and results of restriction of vitamins. There are 78 new illustrations.

## The Conquest of Bacteria‡

A "POPULAR" exposition is *The Conquest of Bacteria*, which can be commended for the general reader, especially because, as stated by Sigerist in the Foreword, it tells the story of chemotherapy in simple and sober terms. It is interesting and informative. A safe book for the public.

## A Manual of Endocrine Therapy§

THE *Manual of Endocrine Therapy* might at first glance give the impression that the author is unduly optimistic about the results in too wide a variety of maladies. In the Summary of Therapy, Chapter 15, we find such subjects as acne, dysmenorrhea, enuresis, frigidity, and homosexualism, with a listing of the appropriate endocrine medications and dosage. However, when one turns to the body of the small book he finds that Dr. Cinberg's discussion of these and similar subjects is adequately critical. In other words, in the summary he presents the recommendations of others, many of which he himself has found to be unwarranted. This is a handy, quick reference manual for therapy which can be well recommended. It is unfortunate that there has been an error in the index so that in most cases the subject referred to appears on the page following that indicated by the index.

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*Biological Symposia. Edited by Howard B. Lewis, Professor of Biological Chemistry, University of Michigan. Vol. V, 247 pages. The Jaques Cattell Press, Lancaster, Pa.

†Textbook of Embryology. By Harvey Ernest Jordan, M.A., Ph.D., Sc.D., Professor of Anatomy and Director of the Anatomical Laboratories, University of Virginia; and James Ernest Kindred, M.A., Ph.D., Professor of Anatomy, University of Virginia. Cloth, ed. 4, 613 pages, $6.75. D. Appleton-Century Company, Inc., New York and London, 1942.

‡The Conquest of Bacteria. From Salvarsan to Sulphapyridine. By F. Sherwood Taylor. Foreword by Henry E. Sigerist. Cloth, 178 pages, $2.00. Philosophical Library, New York.

§A Manual of Endocrine Therapy. By Bernard L. Cinberg, B.A., M.D., Diplomate American Board Obstetrics and Gynecology; Lecturer Obstetrics, New York Polyclinic Medical School and Hospital; Adjunct Obstetrician, Beth Israel Hospital, New York City; Gynecologist, Good Samaritan Dispensary. Cloth, 178 pages, $3.25. Chemical Publishing Company, Brooklyn, N. Y., 1942.